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Title of Thesis: **ANTI-IDIOTYPIC INDUCTION THERAPY FOR THE TREATMENT OF CHRONIC INFLAMMATORY DISORDERS.**
**THERAPEUTIC EVALUATION OF TWO ANTI-SLE A MONOCLONAL ANTIBODIES IN AN ANIMAL MODEL FOR ACUTE INFLAMMATION.**

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# ANTI-IDIOTYPIC INDUCTION THERAPY FOR THE TREATMENT OF CHRONIC INFLAMMATORY DISORDERS. THERAPEUTIC EVALUATION OF TWO ANTI-SLE A MONOCLONAL ANTIBODIES IN AN ANIMAL MODEL FOR ACUTE INFLAMMATION.

By

**AMAL RAHAL**



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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**UNIVERSITY OF ALBERTA**

**FACULTY OF GRADUATE STUDIES AND RESEARCH**

The undersigned certify that they have read, and recomment to the Faculty of Graduate studies and Research for acceptance, a thesis entitled **ANTI-IDIOTYPIC INDUCTION THERAPY FOR THE TREATMENT OF CHRONIC INFLAMMATORY DISORDERS. THERAPEUTIC EVALUATION OF TWO SLE-A MONOCLONAL ANTIBODIES IN AN ANIMAL MODEL FOR ACUTE INFLAMMATION** submitted by AMAL RAHAL in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Pharmaceutical Sciences

DEDICATED

to

The most important people in my life, my husband, Hassan Rahal, and my children, Rima and Ali. Only with their love, sacrifice and patience, was I able to complete this thesis.

## ABSTRACT

The objective of this proposal is the development of a new immunotherapeutic product for the treatment of autoimmune diseases. This project is based on the hypothesis that the inhibition of migration of immunocompetent cells to the site of inflammation would reduce or stop disease progression.

Adhesion molecules expressed on both immune cells and endothelial cells play crucial role in the migration of immunocompetent cells to the site of inflammation and the regulation of these molecules is known to play a critical role in modulating initiation, progression and severity of inflammation. The importance of adhesion molecules in regulating leukocyte extravasation has made these molecules an attractive therapeutic target for the treatment of inflammatory disorders including autoimmune diseases. Among adhesion molecules, selectins and their ligands are particularly attractive targets because they are involved in the initial interaction between leukocytes and endothelial cells and appear to be essential for extravasation of leukocytes into tissues in response to inflammatory stimuli.

Because of their high specificity, monoclonal antibodies represent a particularly attractive tool for the inhibition of selectin function. This project is based on the hypothesis that the injection of a monoclonal antibody (Ab1) directed against a selectin ligand into a host will induce a specific anti-idiotypic response and inhibit leukocyte rolling. The mechanism of action is dependent on the direct effect of Ab1 and more importantly on the effect of the anti-idiotypic (Ab2) and anti-anti-idiotypic (Ab3) antibodies. Those antibodies (Ab1,Ab2 and Ab3) will inhibit the endothelial-leukocyte interaction through the blocking of either selectin ligands or selectins via competitive

inhibition. As therapeutic agents, we have chosen to use monoclonal antibodies directed against $SLe^a$, a carbohydrate antigen recognized by selectins. We have tested the $SLe^a$ specific monoclonal antibodies [HB1 (IgM) and HB2 ($IgG_3$)] in acute inflammation model using one of the most popular methods: carrageenan induced acute inflammation (edema). Inflammation was induced in the rat right hind paw via intraplantar injection with 0.05 ml of 1% carrageenan in 0.9 % NaCL. Paw edema was measured by both Caliper and water displacement methods at regular intervals of time.

Acute inflammation is very fast and strong response in nature, and is difficult to control or prevent. Despite these facts, in all experiments we obtained promising results and it was consistently observed that the groups that have been treated with the anti-$SLe^a$ mAb, HB1 or HB2, have responded with the least inflammatory response measured by both caliper and water displacement measurements ($P > 0.05$).

The therapeutic efficacy of the $SLe^a$ specific mAb, HB1, is likely due to the effect of the induced humoral specific idiotypic response. For the purpose of explaining the mechanism of action responsible for the obtained therapeutic effect, we studied the effect of rat serum on the rolling of human PMNL cells on E- or P-selectin coated channels. Interestingly, the addition of the serum obtained from rats immunized with the HB1 mAb significantly inhibited the rolling of PMNL leukocytes on both E- and P-selectin coated channels. The inhibitory effect of HB1 rat serum on white blood cell rolling was much stronger and more significant the control serum effect. This suggests that there is indeed an induced specific immune response that proves to be responsible for the obtained therapeutic effect. The inflammatory response may involve more than leukocytes, that is why in vitro and in vivo results do not match.

The results obtained encourage further studies to evaluate this approach in chronic inflammatory models.

# ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

## CHAPTER 2. MATERIALS AND METHODS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATION

| | |
|---|---|
| Ab | Antibody |
| AUC | Area under the curve |
| CD | Cluster designation |
| DM | Diabetes mellitus |
| DMARD | Disease modifying antirheumatic drug |
| EDTA | Ethylenediaminetetraacetic acid tetrasodium: hydrate ($C_{10}$ $H_{12}$ $N_2$ $O_8$ $Na_4$) |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FBS | Fetal bovine serum |
| HAMA | Human anti-mouse antibody |
| HUV-EC-C | Human umbilical vein endothelial cells |
| ICAM | Intracellular adhesion molecule |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL | Interleukin |
| IP | Intraperitoneal |
| IV | Intravenous |
| KLH | Keyhole Limpet Hemocyanin |
| LFA | Lymphocyte function antigen |
| mAb | Monoclonal antibody |
| MBP | Mannan Binding Protein |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| MS | Multiple sclerosis |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline tween |
| PMNLL | Polymorphonuclear leukocytes |
| RA | Rheumatoid arthritis |
| RAMA | Rat anti-mouse antibody |
| RGD | Arginine/ Lysine/ Aspartate peptide |
| SC | Subcutaneous |
| SD | Sprague Dawley |
| SLE | Systemic lupus erythematosus |
| $SLe^a$ | Sialyl Lewis a |
| $SLe^x$ | Sialyl Lewis x |
| SM | Standard media |
| TCR | T cell receptor |
| Th | T helper |
| TNF-α | Tumor necrosis factor-alpha |
| TNFR | Tumor necrosis factor receptor |
| VCAM | Vascular cell adhesion molecule |
| VLA | Very late activation |

# CHAPTER 1. INTRODUCTION

## *1.1. Idiotypic network*

### *1.1.1. Jerne's theory*

According to Jerne's network concept (Jerne, 1974), the idiotypic network can be summarized as follows. A given antibody (Ab) (Ab1) reacts with epitopic determinants on an antigen. Although antigenic determinants are mostly found on foreign macromolecules, structural determinants on the variable regions of an Ab1 can also serve as determinants that are recognized by a second Ab (Ab2). The "epitopes" on an Ab1 that are recognized by an Ab2 are called idiotopes, and the Ab2 is an anti-idiotopic or anti-idiotypic Ab bearing the internal image of the antigen. The anti-idiotypic Abs, known as Ab2 may have different specificities based on the region of the variable domain of the Ab1 they recognize (Steinitz et al., 1988). Some recognize the antigen binding site of Ab1 (Ab2β) and resembles the original epitope recognized by Ab1, while others recognize sites present elsewhere on the variable region, which may (Ab2γ) or may not (Ab2α) interfere with the binding site (Steinitz et al., 1988). Furthermore, it was postulated that each anti-idiotype antibody might serve as the antigen for the next anti-idiotype Ab along the cascade (Ab3, etc). By this, Ab2 in turn can serve as surrogate antigen to induce anti-anti- idiotypic Ab (Ab3) that, like Ab1, could potentially bind to the original antigen as illustrated in Figure 1 (Kingsbury et al., 1998). In this way, the antibody cascade could propagate and maintain internal images of the antigen and/or Ab in a persistent autogenic response.

### *1.1.2. Therapeutic approaches based on the idiotypic network theory*

Because of their abilities to act as surrogate antigens, anti-idiotypic antibodies represent a particularly attractive tool for the development of new pharmaceutical agents. Therapeutic approaches based on the idiotypic network can employ either Ab1, a monoclonal antibody (mAb) directed against the targeted antigen, or directly Ab2, an anti-idiotypic mAb which can act as a surrogate antigen.

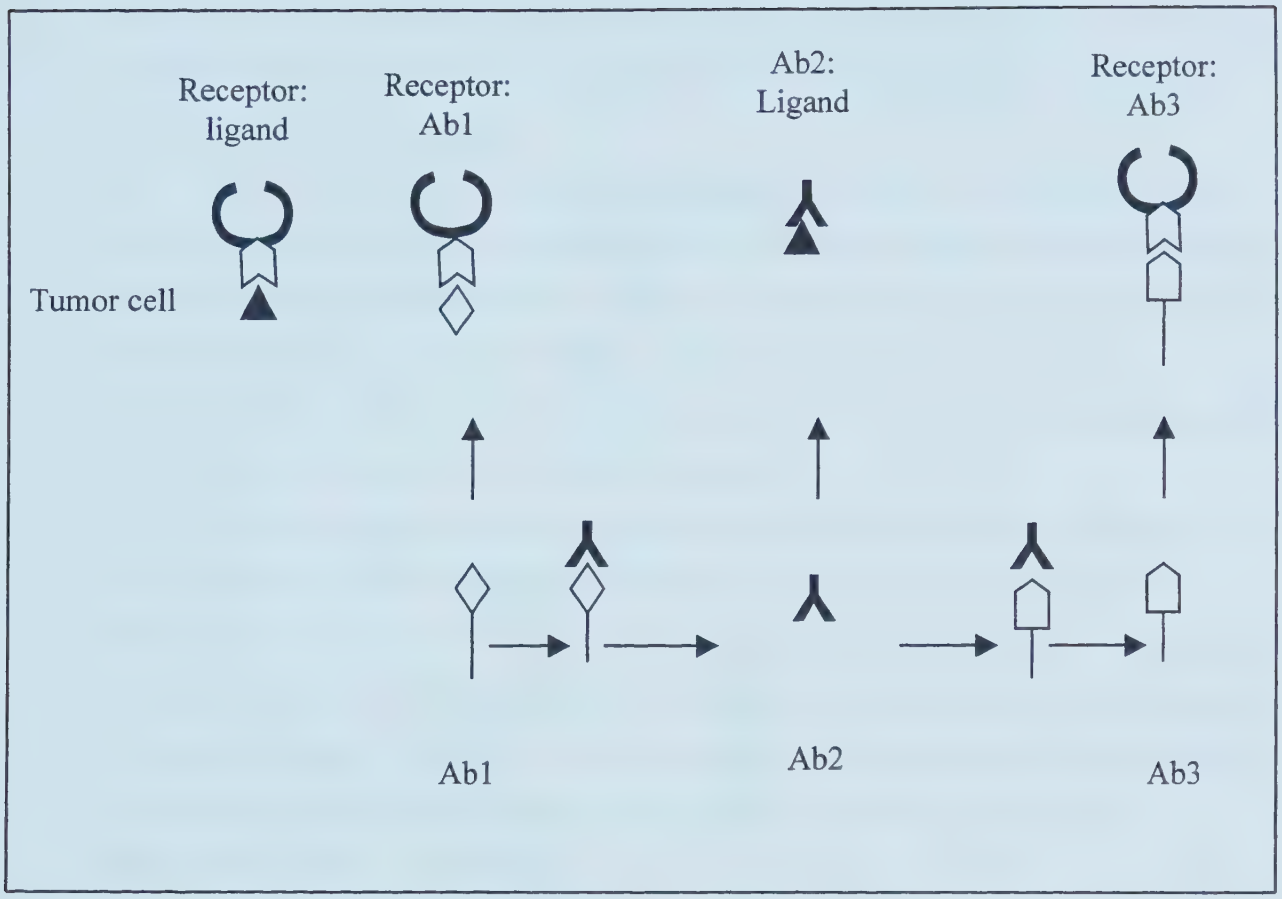



**Figure 1.** Idiotypic cascade showing reactivity with ligand and/or receptor by mirror image. For example, a tumor cell expressing a tumor associated antigen is targeted with (Ab1) that blocks the ligand binding site. The idiotype of this antibody elicits an anti-idiotype respone with generation of Ab2 antibodies. A portion of the Ab2 may mimic receptor topology and also bind ligand. Idiotypes of the Ab2 may in turn elicit Ab3 antibodies. A portion of the Ab3 may mimic ligand topology and bind to receptor (antigen).

The use of Ab1 or Ab2 has several advantages over purified antigens; for example, Ab1 and Ab2 represent a better source of easily producible drugs. They are also safer to use because they do not have tumorigenic or infectious potential. In addition they may be more immunogenic than conventional vaccines particularly in cases where the targeted antigen is a self-antigen or when the targeted antigen is poorly immunogenic (such as carbohydrate antigen).

Ab1 or Ab2 have been used in animal models to induce a protective immunity against a wide range of viral, bacterial, parasitic and tumor antigens (Chapman, 1995). A Number of clinical trials based on the idiotypic approach have also started. Examples of such studies for the treatment of different types of cancer are shown in table 1.
The mechanism of action of Ab1 and Ab2 depends on the induction of both humoral and cellular immune responses as illustrated in figure 2.

The therapeutic action of Ab1 is believed to depend on several mechanisms. The first mechanism of action depends on the ability of Ab1 to induce a humoral anti-idiotypic immune response (Ab2). Ab2 that represents the internal image of the targeted antigen, can in turn induce an anti-anti-idiotypic immune response (Ab3) directed against the targeted antigen. Both Ab1 and Ab3 may lead to antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) and subsequently to tumor cell killing.

The second mechanism of action depends on the ability of Ab1 to form an immune complex with circulating Ag. Numbers of tumor antigens (Ag) are released in the circulation and the presence of circulating targeted antigens is believed to play a crucial role in the therapeutic efficacy of Ab1. Indeed subsequent to the intravenous injection of Ab1, an immune complex will be formed between Ab1 and the targeted Ag. Such immune complex can be processed by antigen presenting cell (APC) (Regnault et al., 1999) leading to the generation of a multiepitopic humoral and/or cellular immune response against the targeted antigen. The binding of Ab1 to targeted Ag may induce a conformational change within the Ag resulting in different processing and the formation of a new peptide for MHC binding (Madiyalakan et al., 1997). The epitope recognized by Ab1 may also be protected and/or processed differently creating a new peptide sequence for MHC binding.

**Table 1. Examples of ongoing clinical trials for human cancers using either Ab1 or Ab3 as therapeutic agents.**

| Cancer | Therapeutic agent | Target antigen | Phase of clinical trial | Reference |
|---|---|---|---|---|
| Gastrointestinal | B72.3 mAb (**Ab1**) | TAG-72 | | Blanco et al., 1997 |
| Gastrointestinal | CC49 mAb (**Ab1**) | TAG-72 | | Blanco et al., 1997 |
| Colorectal | CO17-1A mAb (**Ab1**) | GA733-2 | | Fagerberg et al., 1996 |
| Breast | Brevarex (**Ab1**) | MUC 1 | I | A.A. Noujaim, personal communication |
| Ovarian | B72.3 mAb (**Ab1**) | TAG-72 | | Blanco et al., 1997 |
| Ovarian | CC49 mAb (**Ab1**) | TAG-72 | | Blanco et al., 1997 |
| Ovarian | Ovarex (**Ab1**) | CA125 | II/III | Schultes et al., 1998; Madiyaakan et al., 1997 |
| Melanoma | I-MEL-2 mAb (**Ab1**) (MELIMMUNE-2) | melanoma antigen | I/II | Quan et al., 1997 |
| Non-hodgkin's lymphoma | mAb (**Ab1**) | Autologous B cell lymphoma tumor Ig | | Hsu et al., 1997 |
| Colorectal | 105AD7 mAb (**Ab2**) | 791Tgp72 | I/II | Durrant et al., 1997 |
| Colorectal | (h-Ab2) mAb (**Ab2**) | GA733-2 | I/II | Fagerberg et al., 1995 |
| Colon/lung | CeaVac™ (**Ab2**) | CEA | II/III | phRMA survey, 1997 |
| Breast/ovarian | TriAb™ (**Ab2**) | HMFG | II/III | phRMA survey,1997 |
| Epethelial ovarian | ACA125 mAb (**Ab2**) | CA125 | | Wagner et al., 1997 |
| Melanoma | MK2-23 mAb (**Ab2**) | HMA-MAA | N/A | Mittelman et al., 1995 |
| Melanoma | 4B5 mAb (**Ab2**) | GD2 | I/II | www.novopharmbiotech.ca/cimm |

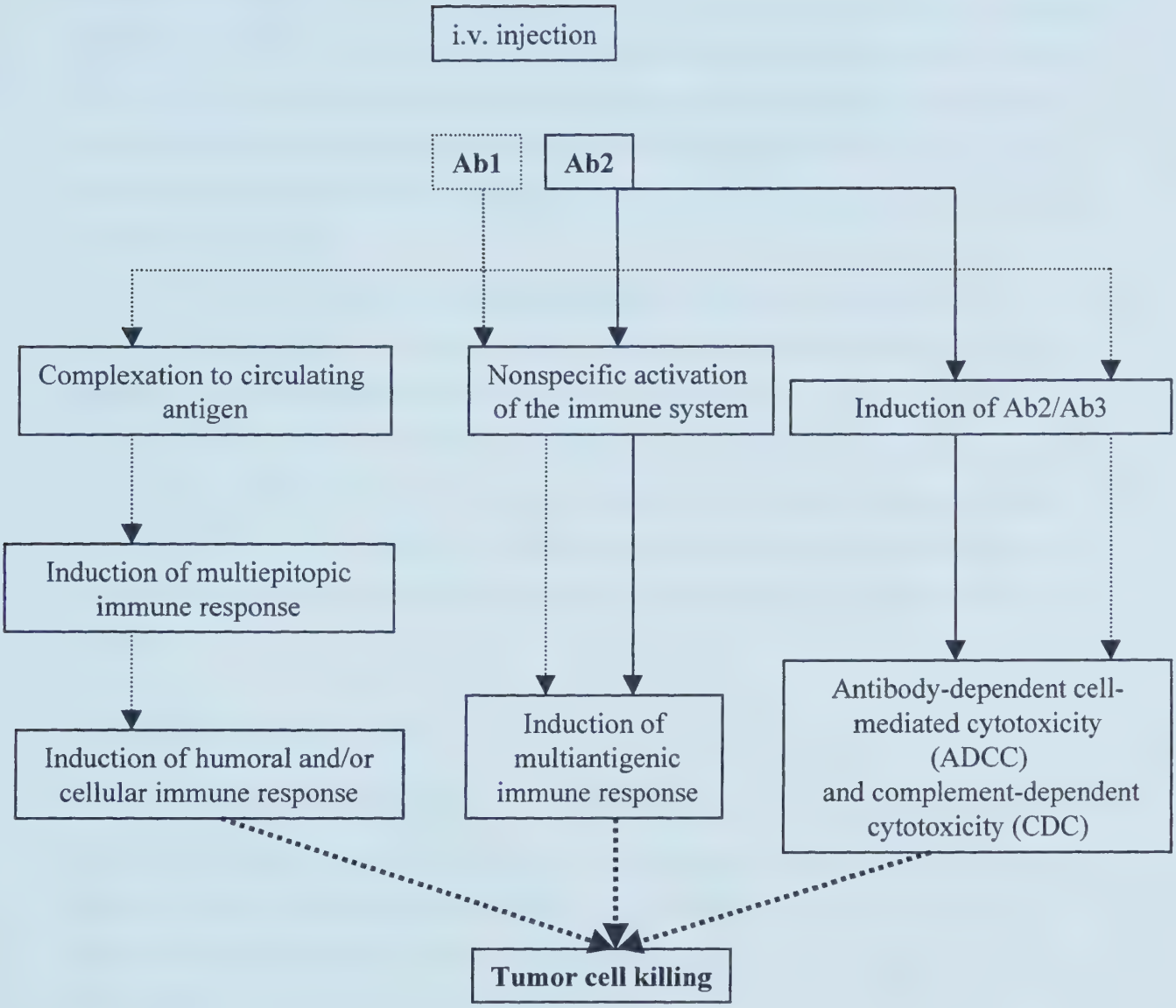



**Figure 2.** Proposed mechanism of action of Ab1 and Ab2 in tumor cell killing through the idiotypic network

AltaRex. Corp. has demonstrated the importance of circulating antigen. Observation in ovarian cancer patients treated with Ovarex (B43.13 mAb, Ab1) showed a better survival that correlates with high levels of CA125 in the sera of patients. Those patients have developed a humoral immune response (Ab2 and Ab3), an Fc-mediated tumor cell killing (ADCC) conducted by anti-CA125 antibodies (Ab3) and a specific cellular immune response (CA125-specific cytotoxic T cells) (Madiyalakan et al., 1997; Schultes et al., 1998).

Finally, Ab1 of murine origin is recognized as foreign and can also nonspecifically stimulate the immune system. Such process may boost the immune response against other tumor antigens leading to multiantigenic immune responses.

The therapeutic action of Ab2 is similar to that of Ab1 with the exception that Ab2 can not complex with the circulating antigen (figure 2). Immunization with anti-idiotypic antibody (Ab2) resulted in the induction of humoral and cellular (T3) immune responses leading to tumor regression in patients with colorectal cancer (Fagerberg et al., 1995, c).

## *1.2. Inflammatory process*

The inflammatory response was described as early as 150 years ago, and was discussed in a series of lectures in 1889 by Cohnheim, who predicted that " it is solely the vessel wall that is responsible for the entire series of inflammatory events". He also concluded that inflammation is the expression and consequence of a molecular alteration in the vessel walls (McIntyre et al., 1997).

Today inflammation is described as a complex protective physiologic response elicited by various stimuli such as infectious agent, localized tissue injury or other trauma (Gallin et al., 1988).

### *1.2.1. Acute versus chronic inflammatory responses*

Acute inflammation is generally accompanied by localized and systemic responses. Acute inflammatory response exhibits rapid onset and is of short duration. The characteristic signs of a localized inflammatory response include swelling, redness, heat, pain and loss of function (Kuby, 1997). In the early stages of an inflammatory response

the predominant cell type infiltrating the tissue is polymorphonuclear leukocytes (PMNL), neutrophils. Neutrophil infiltration into the tissue peaks within the first 6 hours of an inflammatory response, and most of them disappear from the inflamed area within 24-48 hours (Kuby, 1997).

Chronic inflammation develops during persistence of an antigen due to infection or various pathologic conditions. The characteristic hallmark of chronic inflammation is the formation of granuloma. Many different types of cells may be found in the extravascular tissues in areas of chronic inflammation. These include PMNL, eosinophils, macrophages, epithelioid cells, plasma cells, lymphocytes and fibroblasts (Hurley, 1983). In many types of chronic inflammation, lymphocytes form a prominent component of the cellular infiltrate, but the accumulation and activation of macrophages (transformed blood monocytes) is the hallmark of chronic inflammation (Kuby, 1997; Hurley, 1983). Macrophages cooperate with lymphocytes in a variety of ways, and both play an important role in humoral and cell-mediated immunity in chronic inflammation (Hurley, 1983). Studies have suggested that high endothelial venule-like (HEV-like) regions appear along the vasculature in tertiary extralymphoid sites of chronic inflammation and seem to be induced by cytokines that are associated with chronic inflammation. HEV-like regions appear to be the sites of lymphocyte extravasation into inflamed tissues in chronic inflammation. Development of this HEV-like vasculature is likely to facilitate a large-scale influx of leukocytes and particularly lymphocytes contributing to chronic inflammation (Kuby, 1997).

### *1.2.2. Mechanism of leukocyte migration*

The inflammatory response involves numerous mediators and various immune cells. The migration of white blood cells from the blood stream through the endothelial cell wall to sites of injury, infection or immunological reaction is recognized as a characteristic feature and a critical step of the inflammatory response (Issekutz et al., 1996).

The accumulation of leukocytes into inflamed tissues occurs as a consequence of endothelial cell activation and leukocyte migration to the inflammatory foci. The process

of leukocyte migration requires an interaction between leukocytes and the activated endothelial cells (Díaz-González and Sánchez-Madrid, 1998). It is now well established that leukocyte-endothelial cell interaction is a multi-step process, which involves a cascade of sequential cellular adhesive events that can be divided into four successive steps as illustrated in figure 3 (Díaz-González and Sánchez-Madrid, 1998). The initial contact between leukocytes and the blood vessel wall is a reversible process that involves rolling of leukocytes on the activated endothelium, and constitutes the first step in the leukocyte adhesion cascade. Rolling of leukocytes is followed by activation and firm adhesion of leukocytes to the endothelium. In the last step, leukocytes extravasate into the surrounding tissues by squeezing themselves between endothelial cell junctions and moving toward the inflammatory foci (Crockett-Torabi and Fantone, 1995). This multi-step process is facilitated by several species of adhesion molecules present on both leukocytes and endothelial cells as listed in table 2 (McMurray, 1996). Each adhesive event is absolutely important for the whole process of leukocyte extravasation, but rolling of leukocytes is believed to be the initial and most essential step in the development of inflammation. It is of particular importance that neutrophils represent 50-70% of the total circulating leukocytes and constitute the first line of defense. They are the first cells to roll over the endothelium, and the first cells to be recruited through endothelial cells to local sites of infection or injury (Crockett-Torabi and Fantone, 1995).

The general concept of lymphocyte migration is similar to that of neutrophil extravasation, but lymphocytes migrate differentially into different tissues by a process called trafficking or homing. Naïve lymphocytes home to secondary lymphoid organs extravasating across high endothelial venules, while effector lymphocytes home to inflamed vascular endothelium (Kuby, 1997).

### *1.2.3. Selectins and their ligands*

Selectin molecules represent one family of adhesion molecules that play critical role in the adhesive events between leukocytes and endothelial cells. Once the inflammatory response develops various cytokines and other inflammatory mediators induce the expression of cell adhesion molecules including selectins on the vessel wall of

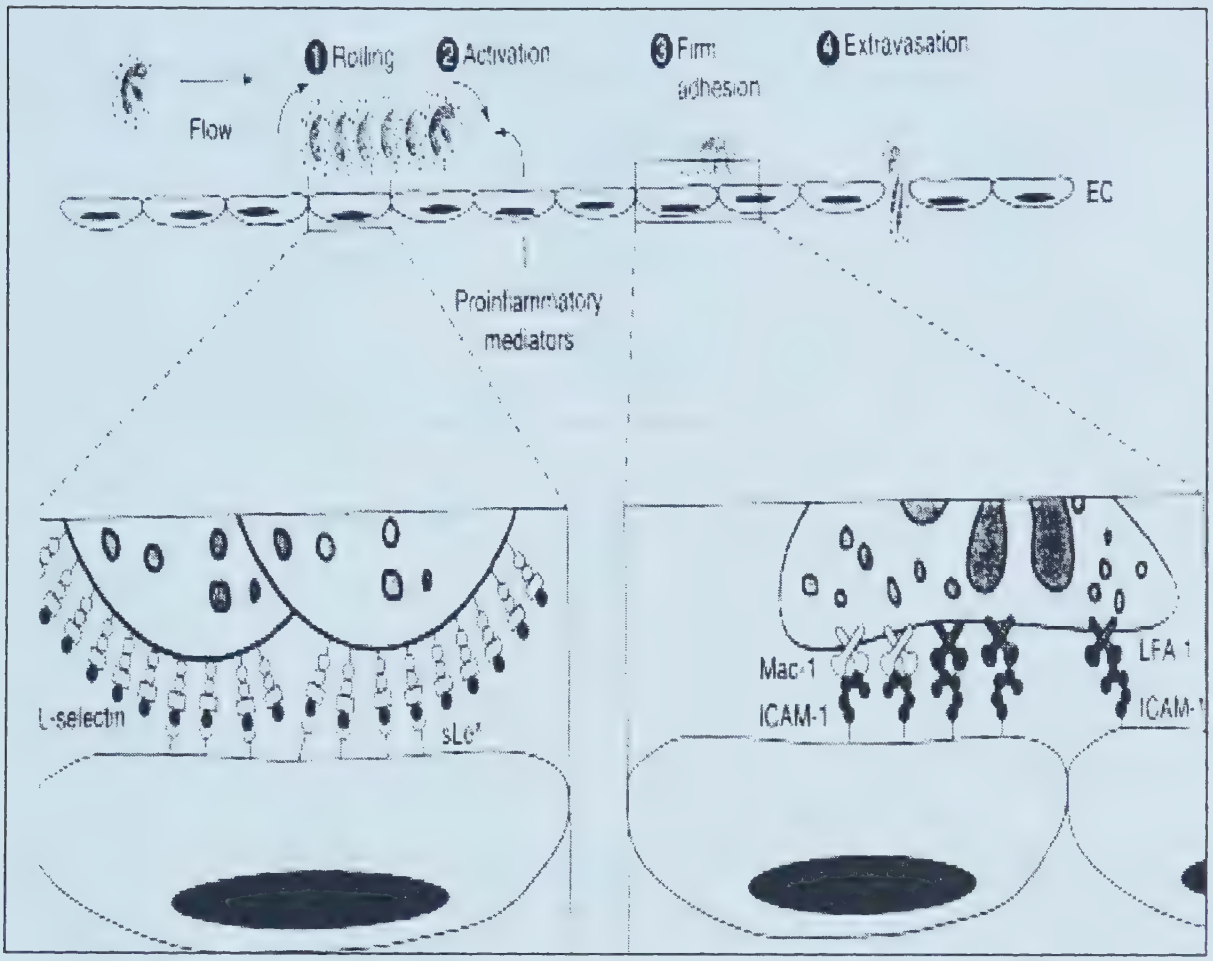



**Figure 3**. Scheme illustrating the different steps in the adhesion cascade between leukocytes and endothelial cells. After tissue injury, circulating leukocytes begin to roll over the endothelial surface (step1). This initial interaction is mediated by members of the selectin family, which recognize mucin molecules expressed on activated endothelium. Leukocytes then became activated by tissue-derived factors (chemokines) that cause changes in the expression level and activation state of specific leukocyte integrins (step 2). The interaction of these activated integrins with their endothelial counter-receptors (ICAM-1) causes the firm adhesion of leukocytes to endothelium(step 3). Finally, leukocytes migrate between endothelial cells into the injured connective tissue (step 4).

## Table 2. Adhesion molecules families.

| Name | Synonym Cluster Designation | Counter-Receptor / ligand | Receptor expression * | Disease Association |
|---|---|---|---|---|
| ***selectin family ( mediate the rolling of leukocyte on endothelial cells)*** | | | | RA, MS, DM |
| L-Selectin | LAM-1, Mel-14 (CD62L) | Glycosylated mucin-like Molecules, Glycam-1, CD34, MadCAM-1 | T cells, monocytes, polymorphonuclear neutrophils, basophil eosinophils | |
| E-Selectin | ELAM-1 (CD62E) | Sialyated mucin-like | Endothelium | |
| P-Selectin | GMP-140 (CD62P), PADGEM | P-selectin glycoprotein ligand (PSGL-1) | Platelets, endothelium | |
| ***Integrin family (mediate the firm adhesion of leukocytes to endothelium)*** | | | | RA, DM, MS, SS |
| $\beta_1$-Integrin | | | | |
| $\alpha_4\beta_1$ | VLA-4(CD49d / CD29) | VCAM, PP-HEV, FN (CS-1) | T, B cells | |
| $\alpha_5\beta_1$ | VLA-5 (CD49e / CD29) | FN (RGD) | T cells, platelets, endothelium, epithelium | |
| $\alpha_6\beta_1$ | VLA-6 (CD49f / CD29) | LM | T cells, platelets | |
| $\beta_2$-Integrins | | | | |
| $\alpha_l\beta_2$ | LFA-1 (CD11a / CD18) | ICAM-1 ICAM-2 | Leukocytes | |
| $\alpha_m\beta_2$ | MAC-1, CR3 (CD11b / CD18) | ICAM-1 C3bi, FN, Factor X | Monocytes, neutrophils | |
| $\alpha_x\beta_2$ | p 150, p 95, CR14 receptor | FN | Monocytes | |
| Additional integrins | | | | |
| $\alpha_4\beta_7$ | LPAM-1 | MadCAM-1, VCAM | T, B cells | |
| ***Immunoglobulin Family (mediate prolonged and firm adhesion of leukocytes to endothelium)*** | | | | RA, SLE, MS |
| ICAM-1 | CD45 | $\alpha_L\beta_2$, $\alpha_M\beta_2$ | All cells | |
| ICAM-2 | CD102 | $\alpha_L\beta_2$<br>$\alpha_4\beta_1$ | Endothelium<br>Endothelium | |
| LFA-2 | CD2 | LFA-3 | T cells | |
| LFA-3 | CD58 | CD2 (LFA-2) | T cells | |

Abbreviations: PP-HEV, peyer's patche-high endothelial venules; FN, fibronectin; CS-1, connecting segment-1; LM, laminin; RGD, arginine/lysine/aspartate peptide; FX, factor X; RA, rheumatoid arthritis; DM, diabetes mellitus; MS, multiple sclerosis; SS, sjogren's syndrome; SLE, systemic lupus erythematosus; VCAM, vascular cell adhesion molecule; ICAM, intracellular Adhesion molecule; LFA, lymphocyte function antigen.

- Constitutive or induced expression.

endothelial cells, and as a consequence the endothelium becomes activated. Upon their expression, selectins mediate the initial attachment of leukocytes to endothelium (rolling) at sites of tissue injury and inflammation which is a prerequisite for subsequent integrin-dependent adherence and transendothelial migration.

Selectins are subclassified according to the cell type on which they were originally identified. E-selectin (ELAM-1, CD62E) (Bevilacqua and Nelson, 1993) is transiently expressed on activated endothelium several hours after stimulation by IL-1 or TNF-α (Yamada et al., 1998). P-selectin (PADGAM, CD62P) (Bevilacqua and Nelson, 1993) is found in α granules and Weibel-Palade bodies of platelets, and vascular endothelial cells and can be rapidly redistributed to the cell surface after stimulation by several mediators such as thrombin and histamine (McIntyre et al., 1997; Yamada et al., 1998). L-selectin (CD62L) (Bevilacqua and Nelson, 1993) is constitutively expressed on circulating neutrophils, monocytes, basophils, eosinophils, and most lymphocytes and was initially identified as a homing receptor for lymph nodes (Whitcup et al., 1997). L-selectin is rapidly shed by proteolytic cleavage following cell activation (Crockett-Torabi and Fantone, 1995). Soluble adhesion molecules including selectins have also been described and they potentially regulate adhesion interaction by competitive binding to ligands or counter-receptors. Soluble adhesion molecules are elevated in inflammatory diseases however their pathophysiological and clinical significance has not been established (Gearing and Newman, 1993).

E-selectin mediates binding of natural killer (NK) cells, memory T cells and monocytes to endothelial cells, while P-selectin mediates binding of monocytes and some lymphocytes to activated endothelium (Crockett-Torabi and Fantone 1995). It is of particular importance that all selectins mediate adhesive interactions between neutrophils and activated endothelium because as we mentioned previously, neutrophils are the first cells to be recruited to sites of infection or injury. The initial interactions between neutrophils and endothelial cells are loose and reversible, and operate under conditions of shear flow. The structure of selectins and their ligands makes them uniquely suited for supporting the type of bond formation and dissociation that must prevail in order for leukocytes to be able to roll under conditions of flow (Crockett-Torabi and Fantone 1995; Rossiter et al., 1997).

Selectins are carbohydrate binding adhesion molecules that share a common structural component consisting of a $Ca^{(2+)}$ dependent N-terminal lectin binding domain (McMurray, 1996). They recognize specific carbohydrate structures on ligands that are composed of sialic acid, fucose, galactose, mannose, and/or an anionic sulfate or phosphate ester moieties such as sialyl Lewis$^x$ ($SLe^x$), sialyl Lewis$^a$ ($SLe^a$), and related structures (McEver, 1994). Other carbohydrate antigens can also serve as ligands for selectins.

The carbohydrate ligand, $SLe^x$ [ NeuAcα2-3Galβ1-4(Fucα1-3) GlcNAc-] on leukocytes (Munro et al., 1992), is recognized by all selectin adhesion molecules. Cell surface $SLe^x$ was found in abundance on nearly all isolated PMNL and monocytes, and at low levels on a substantial portion (up to 40%) of natural killer cells. This carbohydrate moiety was expressed also on approximately 10% of peripheral blood T cells (Munro et al., 1992). It is also documented that endothelial cells have the ability to synthesize $SLe^x$ on sialylated N-acetyllactosamine via the action of alpha (1,3) fucosyltransferase (Renkonen et al., 1997). The $SLe^x$ related epitope, $SLe^a$ [ NeuAcα2-3Galβ1-3(Fucα1-4) GlcNAc-] (Zhang et al., 1997) is recognized by E and P-selectins (Paavonen and Renkonen, 1992). Some studies have suggested that $SLe^a$ might also be an L-selectin recognizing unit (Paavonen and Renkonen, 1992). $SLe^a$ is absent on most peripheral blood cells suggesting that it might have limited relevance for normal physiological condition (Takada et al., 1991).

Selectin-carbohydrate ligands need to be expressed at the appropriate place and at the appropriate time for the relevant binding with selectins (Toshiyuki et al., 1997). The rapid formation and breaking of this ligand pair and the density of expression of the ligands determine the velocity at which the cells roll along the endothelium (McIntyre et al., 1997). The role of selectins in tethering was demonstrated in vivo in P and L-selectin deficient mice, in which leukocyte rolling along the endothelial cells was significantly impaired after surgical stimulus (Ley et al., 1995). It was also shown that in leukocyte adhesion deficiency (LAD) Type II, the congenital absence of selectin ligands produces significant adhesive defects and recurrent life threatening infection (Etzioni et al., 1992).

## *1.3. Clinical and preclinical treatment modalities for RA and other chronic inflammatory diseases*

Excessive inflammation, either secondary to abnormal recognition of host tissue as "foreign" or deviation from an otherwise normal inflammatory response, leads to inflammatory disease (Gallin et al., 1988). Inflammatory diseases have a complex etiology that has been variously assumed to be genetic, infectious or both (Zimmermann, 1997). Regardless of their etiology, most forms of acute and chronic inflammation are amplified as well as propagated as a result of the recruitment of humoral and cellular responses (Gallin et al., 1988).

The majority of inflammatory disorders are frequently chronic in duration, and of particular importance are the autoimmune diseases. Autoimmune diseases is a group of chronic diseases, in which the immune system not only fail to perform its regular function in protecting the host from invading agents, but also reacts particularly against some of the host autoantigens. This autoimmune response is initiated by cellular, humoral, or both mechanisms, and leads to disease with local inflammation and tissue damage (College lecture, 1996). Multiple sclerosis (MS), systemic lupus erythematosus (SLE), Crohns disease, and rheumatoid arthritis (RA) are just few examples from a vast list of autoimmune diseases.

RA represents one of the most common human autoimmune diseases, which occurs in 0.5-2% of the general population (Choy et el., 1998). RA is a systemic chronic inflammatory disease of unknown cause, characterized by symmetric, erosive, and proliferative synovitis, which often leads to joint destruction (American College of Rheumatology, 1996). It is the most common inflammatory and destructive arthropathy in the world, and causes substantial morbidity, and loss of productivity. Furthermore, RA is not only a disabling disease, but also associated with increased mortality especially in its more severe and systemic form (Van de Putte et al., 1998; Choy et al., 1995), therefore more acceptable treatment regimens for RA are in great demands.

### *1.3.1. Agents available for the treatment of RA*

At present, drugs used for the treatment of RA include first and second-line drugs. First-line drugs include salicylates and nonsteroidal anti-inflammatory drugs (NSAIDS). Second-line drugs include disease modifying antirheumatic or slow acting antirheumatic drugs (DMARDs or SAARDs respectively), and corticosteroids (Barrera et al., 1996). The SAARDs is a heterogeneous group that includes antimalarials, thiol compounds, sulfonamides, and antimetabolites, as well as cytotoxic and immunosuppressive agents (Barrera et al., 1996).

However, antirheumatic drugs have also been classified lately into two categories, symptom-modifying antirheumatic drugs (SM-ARDs) and disease-controlling antirheumatic drugs (DC-ARDs). By the new classification, NSAIDs and corticosteroids are categorized as SM-ARDs; second line drugs depending on their effects on radiographic progression and functional outcome, could be assigned to either the SM-ARDs or DC-ARDs (Van de Putte and Van Reil, 1995).

NSAIDS have analgesic as well as anti-inflammatory effects through their inhibition of cyclooxygenase 1 and/or 2, and the reduction of prostaglandin synthesis (Sprangler, 1996). NSAIDS also seem to exert their anti-inflammatory properties, at least in part, by inhibiting rolling of leukocytes along the endothelium as a consequence of a rapid cleavage and shedding of L-selectin from the neutrophil surface (Díaz-González and Sánchez-Madrid, 1998). Glucocorticoids affect virtually every cellular and humoral mechanism involved in inflammation and immune response including cytokines.
The mechanism of action of DMARDs is still poorly understood, but they seem to interfere with cellular metabolic processes and have nonspecific anti-inflammatory, immunoregulatory, and anti-angiogenic effects (Barrera et al., 1996). It is known that several DMARDs, modulate the cytokine network, such as suppressing the proinflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) (Sperber et al., 1993; Awad et al., 1995; Malfait et al., 1993), and Tumor necrosis factor- alpha (TNFα) (Malfait et al., 1993).

Although NSAIDs relieve symptoms in RA, they do not alter the course of the disease, or prevent joint destruction. They have several adverse effects on the gastrointestinal mucosa, kidney, and hemostasis that may be as severe as the side effects of DMARDs (American College of Rheumatology, 1996). Corticosteroids are effective in relieving symptoms of RA, but their chronic use is limited and discouraged because of their adverse effects, which increase with higher doses and longer duration of treatment (American College of Rheumatology, 1996). DMARDs have been the main treatment in RA to date. They have the potential to reduce or prevent joint damage and radiological progression of the disease (Choy et al., 1995) but most of them fail to halt bone and joint destruction, or cure the disease (Kahn, 1995), and therefore DMARD therapy should be continued at a maintenance dose. Unfortunately, DMARDs has a high incidence of toxicity, which requires careful monitoring. DMARDs toxicity interferes with their prolonged administration, restricts the maintenance dose needed during disease remission, and limits the number of patients to be treated with the original drug for long periods.

Currently there is emphasis on combination therapy with DMARDs, such as methotrexate and chloriquine, with attempts to reduce dosage, as well as toxicity, and eventually to increase efficacy (O'dell, 1997). This strategy was not found useful for some combined drugs and there was no conclusive evidence about their additive or synergistic benefit (Paulus, 1993). The combination of methotrexate with cyclosporine had favorable result so far (Tugwell et al., 1995), but a long-term follow up is still needed in order to assess the clinical benefit against toxicity.

Despite the optimal use of all the available antirheumatic drugs, the outcome for many RA patients consists of pain, severe functional decline, and premature death. For these reasons, there is an urgent need for a more efficacious and less toxic therapeutic approaches.

### *1.3.2. New agents being evaluated in clinical trials for the treatment of RA*

A considerable effort is being focused on developing new forms of therapy for the treatment of RA which can modify the course of the disease, with the goal to arrest the

disease process, induce a long term remission or even complete cure of this serious disease. Many studies have been carried out to formulate new therapies for RA but the majority of them have been directed to evaluate anti-cytokine treatment. Advances in biotechnology have made the use of monoclonal antibodies (mAbs) a reality to serve this purpose in clinical trials.

To date, IL-1, TNFα, IL-6 and Interleukin-2 (IL-2) have been the target of specific anticytokine approaches in RA, but the most attention has been drawn by therapies counteracting the two pivotal mediators TNFα and IL-1.

Two anti-TNF-α mAbs were used for the treatment of RA, a chimeric IgG3 mAb, $cA_2$ (centocor), and a human IgG4 mAb, CDP571 (Celltech Therapeutics Ltd, also known as Bay103356, Bayer Corp.). In placebo-controlled trials (Elliot et al., 1994, a; Rankin et al., 1995, a) both mAbs produced dose related clinical improvement but repeated treatments were necessary to maintain disease improvement (Elliot et al., 1994, b). Unfortunately, several side effects were documented including the development of autoantibodies like anti-double stranded deoxyribonucleic acid (anti-dsDNA) and anti-cardiolipin antibodies (Elliot et al., 1994, a; Rankin et al., 1995, b). The anti-TNF-α mAb, $cA_2$ was also used in combination with methotrexate in which results showed that methotrexate at low dose suppressed the antibody response to $cA_2$ (Maini et al., 1998). These drugs are now approved hence large clinical studies are done.

Murine anti-IL-6 mAb (B-E8, IgG1) (Wendling et al., 1993), anti-IL-2 Receptor (anti-IL-2R) mAb (Campath 6) (Kyle et al., 1989) were evaluated for the treatment of RA over a period of 10 days. The treatment with either anti-IL-6 or anti-IL-2R mAb has led to a transient clinical improvement. A clinical trial of a humanized anti-IL-6 mAb treatment in RA is currently under way.

In addition to mAbs, there are other agents that have been used in RA clinical trials to inhibit proinflammatory cytokines. For example, treatment with TNFR: Fc (Enbrel by Immunex), a recombinant fusion protein that consists of the soluble TNF receptor (p75) linked to the Fc portion of human IgG1 (Moreland et al., 1997, b) led to a significant dose related clinical improvement. Unfortunately, cessation of TNFR: Fc therapy was associated with an increase in disease activity, suggesting that continued administration of TNFR: Fc is essential for a sustained effect. Clinical trials are now in

progress to assess the safety and efficacy of Enbrel combined with methotrexate for the treatment of refractory RA, as well as to compare Enbrel to methotrexate in treatment of early RA (Moreland, 1998). Treatment with another recombinant human TNFR (p75)-Fc fusion protein (Lenercept) developed by Roche (Moreland et al., 1997, c) showed clinical improvement but a long-term follow-up is still needed.

Anti-inflammatory mediators such as interleukin-10 (IL-10) (Maini et al., 1997), and IL-1 receptor antagonists (Bresnihan, 1996; Bresnihan and Cunnane, 1998) have been administered to RA patients in clinical trials, and they have shown to cause amelioration of arthritis to varying degrees.

The studies with mAbs or other agents have shown that it is possible to develop rational therapy for RA. Up till now, a curative treatment is still beyond what has been achieved with such therapies and further evaluation is necessary to establish their success rate.

### *1.3.3. New agents under development for the treatment of RA and other autoimmune diseases*

Fortunately, the understanding of the pathogenesis of autoimmune diseases and particularly RA has recently advanced immensely. At the same time molecular technology has made progress in identifying distinct cell subsets, cell surface markers and key mediators of the immune- mediated inflammatory response associated with such diseases. Together, those achievements have opened up the opportunity for new approaches directed towards controlling the production and activity of specific factors involved in the immune response pathway of RA as well as other autoimmune disorders. These approaches were aimed to maximize the benefits and minimize the adverse effects of the applied treatment. The new approaches included oral tolerance, gene, anti-cytokine, anti-lymphocyte, and anti-adhesion therapies.

### *1.3.3.1. Oral tolerance therapy*

Oral tolerance is a condition of immune hyporesponsiveness induced by oral or mucosal exposure to antigens. This state is dependent on the dose of the administered antigen, with a low dose stimulating T cells in the gut. T cells reactive with the ingested antigen migrate from the gut epithelium via lymph channels into the circulation and to the inflamed tissues. At sites of inflammation when T cells encounter the tissues containing the administered antigen, they provide a signal for the secretion of cytokines with a predominant T helper (Th2 and Th3) responses such as, transforming growth factor beta (TGF-β), IL-4 and IL-10 leading to an active immune suppression. On the other hand, high dose oral antigens lead to clonal deletion and anergy (Maini, 1995; Koh, 1998). The encouraging results in animal models with experimental autoimmune diseases such as autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and insulin-dependent diabetes have prompted an interest in oral tolerance therapy as treatment for autoimmune diseases (Koh, 1998). Oral tolerance therapy was evaluated in clinical trials for the treatment of RA patients with either a solubilized chicken type II collagen (Trentham et al., 1993), or bovine collagen (Sieper et al., 1996). Although treatment in both trials showed no side effects, in the first trial statistically significant improvements of disease activity were noted, while in the second one only a minority of patients exhibited clinical response. Larger multicenter controlled trials evaluating the efficacy of oral administration of type II collagen in RA are in progress.

### *1.3.3.2. Gene therapy*

Gene therapy provides another route for treating autoimmune disorders via gene transfer or gene expression. It provides the opportunity to deliver or to increase the local expression of different anti-inflammatory molecules within a defined anatomical location through the use of vectors. This approach has been tested in animal models. For example, genes encoding either interleukin-1 receptor antagonist (IL-1Ra) (Otani et al., 1996; Makarov et al., 1996; Bakker et al., 1997), interleukin soluble receptor (IL-1sR), or TNF soluble receptor (TNFsR) (Ghivizzani et al., 1996) have been transferred into the

synovium of arthritic animals and they resulted in an anti-arthritic effect. Gene therapy has also been carried out on mice with experimental encephalomyelitis, a model of multiple sclerosis, in which retroviral transduction of T lymphocytes with IL-4 complementary DNA (IL-4cDNA) reduced the disease activity (Shaw et al., 1997). Although there are many technical problems in gene therapy that remain to be solved, data from preclinical studies have engendered significant optimism about the future of gene therapy in autoimmune diseases. Most attention, however, has been directed toward treatment of RA. Prospect studies of gene therapy in RA using recombinant IL-1Ra (rIL-1Ra) (McCarthy, 1996) are now in progress.

#### *1.3.3.3. Anti-cytokine therapy*

As cytokines are important mediators of the inflammatory and immune processes, establishing their role in autoimmune diseases was of interest. The importance of cytokine involvement in the pathogenesis of RA was proved by the fact that several cytokines and cytokine receptors were present in RA synovial tissues. For this reason, RA with its accessible lesions, was the ideal disease study. Therefore the anticytokine approaches were evaluated mostly for the treatment of RA. We have previously reviewed, in section 1.3.2, most of the anticytokine therapeutic approaches that have been or being developed in clinical trials for the treatment of RA. In addition, anticytokine therapeutic approaches have also been evaluated for the treatment of other autoimmune diseases such as Crohn's disease, an autoimmune bowel inflammatory disease (Van Deventer and Camoglio, 1996).

#### *1.3.3.4. Anti-lymphocyte therapy*

Clinical research studies designed to intervene at the level of T cells have been pursued where a number of anti-lymphocyte mAbs such as anti-cluster designation (anti-CD) Abs have been used in the treatment of RA. For example, Campath-1H, a humanized anti-CDw52 mAb (Isaacs et al., 1992), the murine mAbs, anti-CD5 (CD5-PLUS) (Strand et al., 1993), anti CD7 (Kirkham et al., 1991; Kirkham et al., 1992), anti-CD25 (anti-IL-

2R) (Banerjee et al., 1988), and anti-CD4 (cM-T412) (Horneff et al., 1991; Wendling et al., 1991) were used for the treatment of RA and other autoimmune diseases such as systemic vasculitis. The initial results in this area held some hope, but they generally have been disappointing. Some of these mAbs failed to result in a clinical improvement of the disease, and some caused high toxicity and unacceptable level of immunosuppression such as profound lymphopenia and depletion of naïve and mature CD4 + T cells. A placebo-controlled trial of a primatized non-depleting anti-CD4 mAbs (IDEC-CE9.1) was carried out in RA (Solinger et al., 1994). Although the initial results of these non-depleting mAbs were encouraging, further placebo-controlled studies with repeated treatments are necessary to assess if they lead to a sustained clinical improvement. Current trials are examining different therapeutic approaches of anti-lymphocyte therapy designed to nonspecifically or specifically block major histocompatibility complex (MHC) or T cell receptor (TCR) function, such as TCR vaccination (Moreland et al., 1997, c). One of the most recent trials using combination of three peptides derived from V beta 3, V beta 14, and V beta 17, has yielded promising results (Bridges and Moreland, 1998). Larger clinical efficacy and safety studies must be performed to determine if TCR peptide vaccination will become available alternative for treatment of RA patients.

#### *1.3.3.5. Anti-adhesion therapy*

Recent reviews have emphasized the role of adhesion molecules in the pathogenesis of RA and other autoimmune diseases (Pitzalis et al., 1994). Cell adhesion molecules and their complementary ligands expressed on endothelial cells, on the surface of circulating leukocytes and lymphocytes play a critical role in controlling the multistep process of leukocyte-endothelial cell interaction. They regulate leukocyte movement across the vascular endothelium into tissues in inflammatory process (Frenette and Wagner, 1996). Leukocyte infiltration into tissues is believed to be a major component of the pathologic process leading to joint injury in chronic arthritis, including rheumatoid arthritis. Therefore, adhesion molecules represent another attractive immunotherapeutic target for intervention in RA and other autoimmune diseases.

Selectins and their ligands have been considered very important targets in inflammatory conditions because of their important role in the initial contact between leukocytes and the vascular endothelium at sites of inflammation.
Blocking selectins or their ligands with antibodies or oligosaccharides has proven to be beneficial in various animal models of inflammation as it is demonstrated in table 3. For example it was demonstrated that in rats that underwent 4 h of hindlimb tourniquet ischemia, the administration of anti-P-selectin mAb, PB1.3, reduced hindlimb injury, but not leukosequestration (Weiser et al., 1996). On the other hand, the anti-P-selectin mAb PB1.3 reduced infarct size by approximately 38% (2 mg/kg) and 28% (5 mg/kg) in rabbits undergoing 30 minutes of ischemia followed by 5 h reperfusion (Yamada et al., 1998). The cardioprotective effect of PB1.3 in this study was explained by the ability of PB1.3 to inhibit polymorphonuclear leukocyte adhesion to coronary endothelium. PB1.3 also preserved coronary flow and myocardial contractile function in a canine myocardial ischemia and reperfusion model (Chen et al., 1994).

Similarly, the anti-rat-P-selectin mAb, ARP2-4, attenuated infarct size in rat myocardial ischemia and reperfusion injury model (Tojo et al., 1996). It has been recently demonstrated that anti-rat-P-selectin mAb, ARP2-4, reduced the footpad swelling and inhibited the associated PMNL accumulation when administered to rats who had undergone dermal injury induced by Arthus reaction (Ohnishi et al., 1996).

Reduction in infarct size as well as PMNL accumulation was observed with the use of anti-E-selectin mAb, BBIG-E5, in a rodent model (Altavilla et al., 1994).
A study on endotoxin-induced uveitis in mice (Whitcup et al., 1997) showed that neutrophil migration into the eye was effectively inhibited with a combination of anti-E-selectin and anti-P-selectin monoclonal antibodies administered intraperitoneally, either before or after endotoxin injection. In this study, anti-E-selectin antibody alone had little effect on endotoxin-induced uveitis, while anti-P-selectin mAb did decrease ocular inflammation by 37% when administered at the time of or 6 hours after endotoxin injection. These data suggest that blocking both E- and P-selectin can effectively reduce inflammatory cell recruitment despite expression of other adhesion molecules.

Reduction in infarct size as well as PMNL accumulation was observed with the use of anti-L-selectin mAb, DREG-200, in a feline model (Ma et al., 1993). In an in vivo

**Table 3. Anti-selectin agents tested in preclinical studies for the treatment of various inflammatory diseases**

| Anti-selectin agent | Specificity | Inflammatory animal model | Observed effect | Reference |
|---|---|---|---|---|
| Anti-P-selectin mAb (PB1.3) | P-selectin | rat hindlimb ischemia | Reduction in hindlimb injury, no reduction in leukosequestrarion | Weiser et al., 1996 |
| | | rabbit myocardial infarction | Reduction in infarct size by 38% and 28% at 2 &5 mg/ kg dose respectively | Yamada et al., 1998 |
| | | canine myocardial ischemia | Preservation of coronary flow and myocardial contractile function | Chen et al., 1994 |
| Anti-P-selectin mAb (ARP2-4) | P-selectin | rat myocardial ischemia | Attenuation of infarct size | Tojo et al., 1996 |
| | | rat dermal injury induced by arthus reaction | Reduction in footpad swelling, inhibition of PMN leukocyte accumulation | Ohnishi et al.,1996 |
| Anti-P-selectin mAb | P-selectin | mice endotoxin-induced uveitis | Decrease in ocular inflammation by 37% with ip administration at the time or 6 hrs after endotoxin injection | Whitcup et al., 1997 |
| Anti-E-selectin mAb (BBIG-E5) | E-selectin | rodent myocardial infarction | Reduction in infarct size and reduction in PMN leukocyte accumulation | Altavilla et al., 1994 |
| Anti-E-selectin mAb | E-selectin | mice endotoxin-induced uveitis | No effect | Whitcup et al., 1997 |
| Anti E- + Anti-P-selectin Mabs | E- & P-selectin | mice endotoxin-induced uveitis | Effective inhibition of neutrophil migration into the eye with ip administration before or After endotoxin injection | Whitcup et al., 1997 |
| Anti-L-selectin mAb (DREG-200) | L-selectin | feline myocardial infarction | Reduction in infarct size & reduction in PMN leukocyte accumulation | Ma et al., 1993 |
| Anti-L-selectin mAb (MEL-14) | L-selectin | murine experimentally-induced chronic peritoneal inflammation | Reduction of macrophage accumulation by 60 %, lymphocyte accumulation by 90% at 48 hrs, granulocyte influx by 80 % at 6 hrs and by 50 % at 24 & 48 hrs | Pizcueta et al., 1994 |
| $SLe^x$ oligosaccharide | selectins | rat myocardial ischemia | Significant reduction in infarct size, no effect on hemodynamic parameters | Tojo et al., 1996 |
| Soluble $SLe^x$ | selectins | rabbits ear edema and necrosis | Reduction in necrosis and edema with immediatediate treatment upon reperfusion (25 mg/kg infusion over 10 hours), no improv-ement with delayed 4-12 hour treatment | Han et al., 1995 |
| $SLe^x$ analogue (CY-1503) | selectins | canine lung allograft | Reduction in PMNL adhesion, migration & reperfusion injury at 35 mg/ kg iv bolus | Schmid et al., 1997 |
| | | rabbit myocardial infarction | No reduction in infarct size (at 30 mg/ kg dose) | Birnbaum et al., 1997 |
| $SLe^x$ oligosaccharide | selectins | feline myocardial infarction | Inhibition of PMN leukoyte-EC adhesion, reduction of infarct size by 83 % | Buerke et al., 1994 |
| | | canine myocardial infarction | Inhibition of PMNL-EC interaction by 63%, reduction in infarct size by 67% | Lefer et al., 1994 |
| Anti-$SLe^x$ mAb | $SLe^x$ | rat myocardial ischemia | Reduction in the extent of myocardial Infarction, increased expression of $Sle^x$ on endothelial cells upon reperfusion of ischemic myocardial tissue | Yoshinori., 1996 |

murine model of experimentally induced chronic inflammation of the peritoneum, anti-L-selectin mAb (MEL-14) was infused intravenously (iv) to investigate the role of L-selectin in the recruitment of mononuclear leukocytes to chronic sites of inflammation (48 hours) (Pizcueta and Luscinskas, 1994). Results from this study showed a reduced accumulation of macrophages and lymphocytes by 60% and 90% respectively, at 48 hours. Similarly MEL-14 mAb dramatically inhibited granulocyte influx by 80% at 6 hours and by 50% at 24 and 48 hours. These results suggest that L-selectins play an important role in leukocyte extravasation at sites of chronic inflammation.

Not only mAbs were utilized to block selectin interactions, but also selectin-carbohydrate analogues were employed for this purpose. Most of the studies on selectin-carbohadrate ligands have targeted sialyl lewis$^x$ ($SLe^x$) oligosaccharide molecule. The effect of $SLe^x$-oligosaccharide was investigated on rat myocardial ischemia and reperfusion injury (Tojo el al., 1996). In this study, the administration of $SLe^x$-oligosaccharide has caused significant reduction in infarct size without affecting hemodynamic parameters or circulating leukocyte numbers. Furthermore, the effect of a soluble $SLe^x$ on ear edema and necrosis was evaluated in rabbits where the ear vascular supply was occluded for 6 hours, and allowed to reperfuse later (Han et al., 1995). Tissue edema and necrosis were significantly reduced in animals treated with $SLe^x$ (25 mg/kg bolus i.v. followed by 50 mg/kg infusion over 10 h) immediately upon reperfusion or after a 1-hour delay, but not in animals for whom the treatment was delayed 4 or 12 hours. It was suggested that the more immediately available P- and L-selectin participate in this PMNL adhesion/injury process, whereas E-selectin, with its delayed endothelial expression, does not. The inhibitory effect of CY-1503, an analogue of $SLe^x$, was evaluated on PMNL migration and reperfusion injury in canine left lung allografts (35 mg/kg iv bolus) where the recipient contralateral right pulmonary artery and bronchus were ligated (Schmid et al., 1997). Allograft gas exchange and hemodynamics results demonstrated that the group treated with CY-1503 had a reduction in PMNL adhesion, migration, and subsequent reperfusion injury in preserved canine lung allografts. In different study on the other hand, the treatment with CY-1503 (30 mg/kg iv bolus) did not limit infarct size or prevent the "no-reflow" phenomenon in rabbits who were subjected to 30 minutes of coronary artery occlusion and 4 hour of reperfusion

(Birnbaum et al., 1997). Other studies demonstrated that $SLe^x$ oligosaccharide reduced infarct size by 83%, inhibited PMNL adhesion to endothelial cells in the feline model (Buerke et al., 1994), but in the canine model it reduced infarct size by 67% and ameliorated PMNL accumulation in myocardium by 63% (Lefer et al., 1994).
In addition, the effect of anti-$SLe^x$ mAb and the expression of $SLe^x$ were examined in rat hearts subjected to 30 minutes of ischemia followed by reperfusion (Yoshinori et al., 1996). Reperfusion of ischaemic myocardial tissue resulted in enhanced expression of $SLe^x$ on the luminal surface of vascular endothelial cells, as well as myocytes. Furthermore, the in vivo administration of anti-$SLe^x$ mAb significantly reduced the extent of the myocardial infarction.

Several studies have targeted adhesion molecules of the immunoglobulin superfamily (IGSF). For example, in an open-label study 32 RA patients were treated daily for five days with three different doses of BIRR-1, a murine $IgG_{2a}$ anti-intracellular adhesion molecule mAb (anti-ICAM) (Kavanaugh et al., 1994). Patients who were treated with the higher doses of the mAb have achieved significant anti-ICAM-1 mAb concentration in vivo to a level known to inhibit adhesion in vitro, and shown improvement in arthritis activity, but most patients relapsed after three months. The serum concentration of anti-ICAM-1 mAb decreased rapidly after treatment since no circulating anti-ICAM-1 mAb was found 1 week later. As expected with a murine mAb treatment, all patients developed a human anti-mouse antibody (HAMA) response by 15 days. Transient leukocytosis was noted in all patients, which was interpreted as the effect of anti-ICAM-1 mAb to block migration of lymphocytes from peripheral blood into inflammatory sites. Patients have also developed impaired T-cell function with a reduction in lymphocyte proliferation and consequently a diminished cutaneous delayed-type hypersensitivity response to tuberculin PPD during treatment. Allergic side effects were more common. Patients whose disease has relapsed, they were given a repeated anti-ICAM-1 mAb treatment to assess the safety and efficacy of a second course of murine anti-ICAM-1 mAb treatment (Kavanaugh et al., 1997). The second course of therapy was associated with adverse effects that were not seen in the initial course of therapy. These adverse effects included the consumption of serum complement protein, the formation of immune complexes such as, murine anti-ICAM-1 mAb/ HAMA and

circulating ICAM-1/murine anti-ICAM-1 mAb/HAMA. In addition, activation of endothelial cells occurred as a result of HAMA cross-linking to the anti-ICAM-1 mAb bound to the endothelial surface. Clinical efficacy associated with the second course of therapy was less effective than that observed in the first course, in both extent and duration.

The systemic administration of anti-ICAM-1 mAb was also evaluated in experimental immune-mediated labyrinthitis in rats (Takasu and Harris, 1997). This treatment has resulted in reduction of inflammatory cell infiltration in the scala tympani and the perisaccular tissue of the endolymphatic sac. These findings suggest that the use of ICAM-1 antagonist may be a possible anti-inflammatory therapeutic approach to labyrinthitis. None of these studies have reached the clinical trials stage.

Adhesion molecules were also targeted for the treatment of established adjuvant arthritis in rats. Such adhesion molecules included the $\alpha_4$ integrin, very late activation antigen-4 (VLA-4,), and the $\beta_2$ integrins, Lymphocyte function antigen-1 (LFA-1, $CD11_a$/CD18) and macrophage-1 (MAC-1, $CD11_b$/CD18) (Issekutz et al., 1996). It was demonstrated that blocking mAbs to $\alpha_4$ and $\beta_2$ integrins administered alone, or in combination, can reduce the severity of adjuvant arthritis, even after joint inflammation has developed, and can markedly inhibit PMNL and T lymphocyte migration to joints. Studies have been carried out to evaluate the pharmacokinetics, pharmacodynamics immunogenecity and safety of a humanized anti-CD11/CD18 (HU23F2G) monoclonal antibody in patients with multiple sclerosis (Bowen et al., 1998). It was shown that HU23F2G was tolerated at doses which acheived high degrees needed for in vivo inhibition of leukocyte migration. This study was not designed to determine the clinical efficacy of HU23F2G; therefore further studies are needed.

CD44 is a ubiquitous adhesion molecule that was targeted for the treatment of murine arthritis (Mikecz et al., 1995). CD44 preferentially binds hyaluronan, a polysaccharide macromolecule that is present in most extracellular matrices. CD44 is overexpressed by synovial cells and leukocytes and overproduced in the rheumatoid synovium and in other inflammatory sites. The anti-CD44 antibody induced a rapid loss of CD44 from both leukocytes and synovial cells and displayed inhibitory effect on cell-extracellular matrix interactions in the synovium. As a result, the administration of such

an antibody in murine arthritis abrogated tissue swelling and leukocyte infiltration, the two major components of inflammation. Anti-CD44 mAb was also effective in reducing the incidence of collagen type II induced arthritis in mice (Zeilder et al., 1995). In the same arthritis model, the mAb against alpha 4-integrin was even more effective in reducing the visible disease symptoms than the anti-CD44 mAb (Zeilder et al., 1995).

## *1.4. Hypothesis*

It has been already reviewed that the selectin-carbohydrate ligand interaction is responsible for the initial attachment of leukocytes (rolling) to endothelium at sites of tissue injury and inflammation. Based on this observed fact we thought that the induction of a specific anti-idiotypic response capable of blocking selectin-ligand interaction could inhibit leukocyte rolling and therefore be of particular interest for the development of a new therapeutic approach for various inflammatory disorders. We propose that the injection of a selectin ligand specific mAb (Ab1) into a host could inhibit leukocyte rolling through two different mechanisms. The first mechanism is dependent on the direct effect of Ab1 that will act to block selectin ligand on either white blood cell surface or endothelial cell membrane, and makes it unavailable for selectin binding. The effect of Ab1 is a direct but unfortunately is also a short- term one due to the rapid clearance of Ab1 from the circulation. It is documented that Ab1 usually remains in the circulation for several hours or few days only. The second mechanism is dependent on the induction of anti-idiotypic (Ab2) and anti-anti-idiotypic (Ab3) Abs. Since Ab2 mimics selectin ligand it will inhibit through its binding to selectin molecules the interaction between selectins and their natural ligands. Ab3 will act as Ab1 and compete with the naturally existing selectin molecule in binding to carbohydrate ligand and as a result, can inhibit the interaction between selectins and their ligands through competitive inhibition.

The effect of Ab2 and Ab3 or what we may call the indirect effect of Ab1 is likely to be more efficient than the direct effect of Ab1 for the inhibition of leukocyte migration. This is due to the fact that Ab2 and Ab3 antibodies are produced by the host and they remain in the circulation for a considerable period of time usually for several months.

## *1.5. Specific aims*

The aim of this study is to evaluate whether the immunization of a host with an anti-$SLe^a$ mAb can inhibit the inflammatory response induced in rat paw by carrageenan, and induce a specific immune response capable of inhibiting leukocyte rolling in vitro.

# CHAPTER 2. MATERIALS AND METHODS

## *2.1. Materials*

RPMI-1640 media, fetal bovine serum (FBS), L-glutamine, streptomycin, penicillin, trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA.4Na), ABTS $H_2O_2$ solution and ABTS peroxidase substrate were obtained from LifeTechnologies GIBCO BRL (Burlington, ON, Canada). Endothelial cell basal medium modified MCDB 131, hydrocortisone, human epidermal growth factor (HEGF), bovine brain extract (BBE), gentamycin sulfate and amphotericin B were obtained from Clonetics Corp. (CA, USA). Histopaque-1077, trypan blue solution, EDTA, Deoxycholic acid (Sodium salt), thimerosal, Chloramine T (CT), pristane (2,6,10,14-tetramethyldecanoic acid), KLH (Hemocyanin from keyhole limpet), glutaraldehyde, carrageenan lambda type IV (gelatin, vegetable, Irish moss), goat anti-mouse polyvalent Immunoglobulin (IgG, IgA, IgM)-FITC conjugate, goat anti-mouse polyvalent Ig (whole molecule), the clarified ascites containing the mouse IgG3κ monoclonal antibody Flopc-21 and BSA 30% were obtained from SIGMA chemical Co. (St Louis, MO, USA). Goat anti-mouse Ig (H+L)-HRP was obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). The 3'-$SLe^a$ oligosaccharide was obtained from Oxford GlycoSciences Inc. (MA, USA). The Mannan Binding Protein column, the Protein A column and the Detoxi-Gel Endotoxin removing Gel (polymixin B) column were obtained from PIERCE (Rockford, IL, USA). Filters 0.22 μm and 0.45 μm were obtained from Millipore (Bedford, MA, USA). Dialysis membrane (MW cutoff, 12,000-14,000 Dalton) and Sodium iodide were obtained from Fisher Scientific (Nepean, ON, Canada). SDS, Mini-PROTEAN II 2-D cell, 20% Tris-HCl ready gel sandwich, the SDS-PAGE molecular weight standard markers and the Desalting Econo-pac 10 DG chromatographic column were obtained from BIO RAD (Hercules, CA, USA). Glycerol, Tris (hydroxymethyl) methylamine and Sodium metabisulfate were obtained from BDH Inc. (Toronto, ON, Canada). Adjuvant QUIL A was obtained from Calbiochem (SD, USA). The Diaflo ultrafilter membranes YM-30 (MW cutoff 30,000 Dalton) and the ultrafiltration cell- 8200 model were obtained from Amicon Canada Ltd. (Oakville, ON, Canada). All other reagents were of analytical grade or equivalent purity. AltaRex Corp.

(Edmonton, AB, Canada) provided the tumor antigen CA19-9, the polyacrylamide conjugated $SLe^a$ antigen, colo 205 tumor cell line, HB1 and HB2 hybridoma cell lines. Alberta Research Council (Edmonton, AB, Canada) provided the cell rolling microchip device. Recombinant human E- and P-selectin, anti-human E- and P-selectin monoclonal antibodies were obtained from R & D Systems (Minneapolis, MN, USA).
Fresh blood samples were obtained from healthy human volunteers in Jasper laboratories (Edmonton, AB, Canada). Sprague Dawley (SD) rats age 6 weeks, weight 275-300 g and Balb/c mice (6 weeks age) were obtained from Health Sciences Laboratory Animal Services (Edmonton, AB, Canada).

## *2.2. Cell culture*

HB1 is a murine, ascites producing, hybridoma cell line, which secrete IgM mAb directed against the tumor marker, CA19-9. HB1 hybridoma cell line was produced and kindly provided by AltaRex Corp. HB1 hybridoma cells were maintained in standard media (SM) (RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin, 10% v/v FBS).

HB2 is a murine, ascites producing hybridoma cell line, which secrete $IgG_3$ mAb that binds specifically to the tumor marker CA19-9. HB2 cell line was produced and kindly provided by AltaRex Corp. HB2 hybridoma cells were maintained in SM.

F5 is a murine, ascites producing hybridoma cell line, which secrete IgM mAb directed against human prostate antigen. F5 hybridoma cell line was obtained from ATCC (Maryland, USA). F5 hybridoma cells were maintained in SM.

7C2C5C12 is a murine, ascites producing hybridoma cell line, which secrete IgM mAb directed against Trichinella spiralis. The cell line, 7C2C5C12, was obtained from ATCC. 7C2C5C12 hybridoma cells were maintained in SM.

Colo 205 is a human adenocarcinoma cell line derived from metatstatic colon cancer that produces CA19-9 antigen. Colo 205 cells were obtained from ATCC. Colo 205 cells were maintained in SM.

Human umbilical vein endothelial cells (HUV-EC-C) is an endothelial cell line derived from the vein of a normal human umbilical cord, obtained from ATCC. HUV-

EC-C cells were maintained in MCDB 131 media supplemented with 10 ng/ ml of HEGF, 12 μg/ ml of BBE, 1 μg/ ml of hydrocortisone, 5 % v/v FBS, 0.05 mg/ ml of Gentamycin sulfate, and 0.05 mg/ ml of amphotericin B.

## *2.3. Isolation of human white blood cells*

Histopaque –ficoll solution (6 ml) was added to a 15-ml test tube, and then a 6-ml of heparin-treated fresh human blood was carefully layered on top of the gradient. Tubes were centrifuged at 1700 rpm (450 x g) for 35 minutes at room temperature.
The band at the upper interface (mononuclear cells) was retrieved (5 ml) and mixed with 10 ml of 0.45% NaCl and 20 ml of PBS (8 mM Di-sodium hydrogen orthophosphate, 3 mM potassium dihydrogen orthophosphate, 0.14 M NaCl). The tubes were centrifuged at 1500 rpm (400 x g) for 15 minutes at 21 °C. The pellet was retrieved and washed three times with 10 ml of 3 mM EDTA, 1% FCS in PBS solution without magnesium and without calcium and centrifuged at 800 rpm (250 x g) for 10 minutes. The pellet was suspended in 2 ml of RPMI with 5% FCS.

In the case of isolating PMNL the band at the lower interface was retrieved and treated in the same way as with monouclear cells. The purified cell pellet (mononuclear or PMNL cells) was suspended at 1x $10^7$ cells/ml in RPMI media, 5% FCS and kept at 37 °C until used.
Cell viability was determined by exclusion of 0.4% trypan blue.

## *2.4. Isolation of rat bone marrow cells*

The whole leg was obtained from Sprague Dawley rats (age 8 weeks) just after they have been sacrificed. The leg surrounding muscles and tissues were removed and the hip and leg bones were isolated. The bones were cut slightly at the lower and the upper tips to allow visualization of the bone marrow. Then with a syringe, the bone marrow was washed several times with PBS and the wash rich in bone marrow cells was allowed to run into a container. The collected cell wash was first centrifuged at 200 rpm for five minutes. The pellet with unwanted material was disposed and the supernatant was

retrieved and centrifuged at 1500 rpm for 15 minutes. The cell pellet was retrieved and suspended at 1x $10^7$ cells in RPMI media, 5% FCS and kept at 37 °C until used (for maximum of 1 hour). Cell viability was determined by exclusion of 0.4% trypan blue.

## *2.5. Antibody production and purification*

### *2.5.1. Ascites production*

Adult mice (age 6 weeks) were primed by injecting 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) into the peritoneum (ip). Two weeks later the mice were injected ip, with 5 x $10^6$ hybridoma cells resuspended in 0.5 ml of PBS. Ascetic fluid was tapped from mice 2 to 3 times in due time, and the collected fluid was incubated for 1 hour at room temperature and transferred to 4 °C overnight. The fluid was centrifuged at 2000 rpm for 15 minutes and the supernatant was stored at –20 °C.

### *2.5.2. Purification of mAbs*

#### *2.5.2.1. MBP affinity chromatography*

The ascetic fluid containing the IgM was dialyzed against binding buffer (10 mM Tris, 1.25 M NaCl, 20 mM $CaCl_2$, and 0.02 % Sodium azide ($NaN_3$) at a pH of 7.4), after which the ascetic sample was diluted 1: 1 v/v with binding buffer, and filtered with 0.22 μm filter. The MBP (Mannan Binding Protein) column was prewashed with 2 column volume (20 ml) of elution buffer (10 mM Tris, 1.25 M NaCl, 2mM EDTA and 0.02% $NaN_3$), and then equilibrated with 4 column volume (40 ml) of binding buffer. The ascetic fluid (maximum 1.5 ml/ 5 ml of gel) was applied to the column, allowed to completely enter the gel, and incubated at 4 °C for 30 minutes. The column was washed with 9 column volumes of the binding buffer to remove the unbound proteins. The wash was monitored for the presence of proteins by measuring the absorbance at 280 nm, using the binding buffer as a reference. The column was removed from the cold and incubated with the elution buffer at room temperature for 1 hour, after which the column was

washed with the elution buffer, and minimum of 14 fractions were collected each with a volume of 3 ml. The elution of IgM was monitored for the presence of proteins by spectrophotometer at 280 nm using the elution buffer as a reference.
At the completion of the purification the column was washed with 2 column volumes of deionized water and then by 2 column volumes of 4 °C binding buffer and stored at 4 °C.

### *2.5.2.2. Euglobulin precipitation method*

The euglobulin precipitation method is a non-chromatographic method used for the purification of both murine $IgG_3$ and IgM mAbs, which takes advantage of their euglobulin properties. This method was performed as described by (Garcia-Gonzalez et al., 1988).
$CaCl_2$ was added to the ascetic fluid (final concentration, 25 mM) to generate fibrin formation. When the clot was formed, it was removed by paper filtration. Then the filtered ascetic fluid was dialyzed for 2 hours at 20 °C (IgG3), or for 15 hours at 4 °C (IgM) against 100x volume of demineralized water (pH 5.5). The ascetic fluid was centrifuged in a Beckman L8-55 ultracentrifuge at 22,000 x g for 30 minutes and the precipitate was recovered and suspended in 1M NaCl/0.1 M Tris-HCl pH 8. Dialysis and precipitation were repeated twice. Purified pellet with a high lipid content was mixed with 1.7 M NaCl and centrifuged for 3 hours at 27,000 x g. Lipid supernatant was discarded, the clarified mAbs solution was dialyzed against 0.1M Tris HCl, 1M NaCl pH 8, centrifuged at 22,000 x g for 30 minutes. The purified pellet was suspended in PBS.

### *2.5.2.3. Protein A affinity chromatography*

The 5 ml Protein A column was washed with 2 column volumes of elution buffer (0.1 mM Glycine, 1mM NaCl, 0.001 % tween 20, pH 4). Then the column was equilibrated with 5 column volumes of binding buffer (50 mM Tris–HCl, 1mM NaCl, 0.001 % tween 20, pH 8). The ascetic sample containing $IgG_3$ antibody was diluted 1:1 v/v with binding buffer and loaded to the column at a rate of 0.75 ml/ minute, then the column was washed with 50 ml of binding buffer, at a rate of 1.5 ml / minute. The

collected fractions containing unbound proteins were checked by spectrophotometer at 280 nm, using the binding buffer as a reference. The column was washed with 15 ml of elution buffer. Then the eluted fractions were collected at a rate of 1 ml /minute and adjusted to a pH of 7 using 50 mM Tris-HCl. Elution of bound proteins was monitored by spectrophotometer at 280 nm, using the elution buffer as a reference.

### *2.5.3. Removal of endotoxins from purified Immunoglobulins*

A 10 ml Detoxi-Gel column was regenerated by washing the gel with 5 column volumes of 1% sodium deoxycholate. Then the column was washed with 3 column volumes of pyrogen-free water. The purified and concentrated immunoglobulin was loaded to the column, and then the sample was collected by gravity flow using PBS as elution buffer.

### *2.5.4. Concentration of Immunoglobulins*

Before use, the ultrafilter YM-30 membrane (MW cutoff 30,000 Dalton) was floated with distilled water for one hour, changing the water three times. Then the membrane was mounted in an ultrafiltration cell and rinsed with distilled water at 20 psi (3.7 atm) for at least 5 minutes. The samples of purified Igs were loaded in the cell, and the sample flow through the ultrafilter membrane was operated under a maximum pressure of 40 psi (4.7 atm). The Ab solution was concentrated approximately 10x and then washed at least three time using PBS.

### *2.5.5. Quantification of Immunoglobulins*

The quantification of purified F5 IgM, 7C2C5C12 IgM and Flopc-21 $IgG_3$ mAbs was done by ELISA where plates were coated with goat anti-mouse Ig as described in section 2.9.1 and by measuring the absorbance at 280 nm (using $\varepsilon = 0.75$). The quantification of HB1 IgM and HB2 $IgG_3$ mAbs was done by ELISA where the plates were coated with CA19-9 antigen as described in section 2.9.1 and by measuring the

absorbance at 280 nm (using $\varepsilon = 0.75$). For each tested mAb an isotype matched control mAb was used as a standard.

### *2.5.6. Antibodies purity*

SDS- polyacrylamide gel electrophoresis was used to check the purity of each of the purified mAbs. Ab samples were diluted 1:4 v/v with SDS-PAGE sample buffer (0.5 M Tris-HCl at pH 6.8, 20% glycerol, 10% w/v SDS, 10% β- mercaptoethanol and 0.5 % bromphenol blue) and heated for 4 minutes at 95 °C. The Tris-HCL ready gel sandwich (10% acrylamide) was inserted into the Mini-PROTEAN II cell clamp assembly and aligned properly. Then the gel sandwich was attached to the inner core of the Mini-PROTEAN II cell. The upper and the lower buffer chambers were filled with approximately 200 ml of 1x running buffer (0.3% Tris base, 1.45% glycine, 0.1% SDS, pH 8.3). 6 µl of molecular weight markers in sample buffer was applied to one of the wells, while the antibody samples, each with a volume of 30 µl (1.5-5 µg of antibody) were applied to the rest of the wells in the gel. The core assembly was inserted into the lower buffer tank and the gel was run at 200 V constant voltage for approximately 45 minutes. The gel was then removed from the gel sandwich, immersed in staining solution (10% acetic acid, 0.025% Coomassie BlueG-250, methanol 40%) for 1.5 hour, and destained in destaining solution (10% acetic acid, 40% methanol) until the desired destaining was reached.

## *2.6. Fluorescence-activated cell sorter (FACS) analysis and Fluorescence microscopy*

$1x10^6$ white blood cells or HUV-EC-C washed in PBS, 1% bovine serum albumin (BSA), were incubated with primary Ab at a concentration of 5 µg/ ml in PBS, 1% BSA. The incubation was performed at 4 °C and after 45 minutes the cells were washed three times with PBS, 1% BSA. The cells were then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse Ab at 4 °C for 45 minutes and washed three times.

The cells were resuspended in PBS, 1% BSA. Fluorescence associated with the cells was analyzed either by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) or by fluorescence microscopy. As negative controls, cells were incubated according to the same procedure but without primary Ab.

## *2.7. Radiolabeling of HB1 IgM mAb*

50 μg of HB1 mAb was mixed with approximately 600 μci of Na $^{125}$I. 10 μl of 2.5 mg/ml of chloramine-T (CT) was added to the reaction vial and gently shaken for 30 sec. Then a 10 μl of 5 mg/ml sodium metabisulfate (Na-Met) was added to the mixture and shaken gently for 15 sec. Finally, 20 μl of 1M sodium iodide (NaI) was added and the whole mixture was gently shaken for 5 sec.

The reaction mixture was loaded to the Desalting Econo-pac 10 DG chromatographic column pre-washed three times with 0.5% BSA in PBS. The vial was rinsed with 100 μl of PBS and transferred to the column. The column was then washed with 0.5 ml PBS fractions 12 to 14 times. Each elute was collected separately in numbered tubes. The radioactivity of elutes was monitored by a Geiger counter. The fractions containing the mAbs were pooled, labeled, placed in lead containers, and stored at 4 °C.

The quantification of $^{125}$I labeled HB1 mAb in the pooled fractions was done by ELISA as described in section 2.9.1 in which plates were coated with CA19-9. A nonradioactive control HB1 mAb was used as standard.

## *2.8. Animal experiments*

### *2.8.1. Conjugation of antibodies with KLH*

1mg of mAb suspended in PBS solution was mixed with 1 mg of KLH and 2 μl of glutaraldehyde (final concentration, 25%). The mixture was incubated for 1 hour at room temperature and then 75 mg of glycine was added to the mixture. The solution was stirred for 1 hour and then dialyzed against distilled water for three days.

The binding activity of the mAbs to CA19-9 antigen was measured before and after conjugation with KLH and compared by Elisa as described in section 2.9.1 in which plates were coated with CA19-9 antigen.

### *2.8.2. Immunization*

Male Sprague Dawely rats (age six weeks) were divided into three groups. Each group was subjected to repetitive injections either with PBS, control mAb or therapeutic mAb. Details about the sample size, number of immunizations, dose of the injected antibodies, routes of Abs administration, and the form of the injected Abs are shown in table 5. Interval time allowed between each immunization is shown in tables 6 and 7.

### *2.8.3. Serum collection*

Serum collection was routinely done prior to each immunization as indicated in tables 6 and 7. The tails of rats were cut slightly at the tip and the blood was collected in serum separator microtainers. During this procedure the rats were anaesthetized with ethyl ether. The collected blood was centrifuged at 300 x g for five minutes; serum was separated, and used to test for the detection of RAMA and Ab3 responses. The animals were kept under care until inflammation was induced and measured.

### *2.8.4. Induction and measurement of Inflammation*

Inflammation was induced in the rat right hind paw (intraplantary) with 0.05 ml of 1% carrageenan in 0.9 % NaCL, five days after the last immunization as shown in tables 6 and 7. The animals were lightly anaesthetized with ethyl ether for the injections and for inflammation measurements.

Paw edema was measured by two different methods. Caliper method was used to measure paw thickness, and water displacement method to measure paw volume. The thickness of rat paw was measured in inches by a caliper device before and after carrageenan injection at desired time intervals. The paw volume was measured by

## Table 5. Design and characteristics of in vivo experiments.

| | experiment 1 | experiment 2 | experiment 3 | experiment 4 | experiment 5 |
|---|---|---|---|---|---|
| **Number of rats/ group** | 6 | 10 | 8 | 10 | 10 |
| **Therapeutic (T) and control (C) antibodies** | HB1 (T)<br>F5 (C) | HB1 (T)<br>F5 (C)<br>7C2C5C12 (C) | HB1 (T)<br>F5 (C) | HB1 (T) | HB2 (T)<br>Flopc21 (C) |
| **Antibody isotype control and therapeutic** | IgM | IgM | IgM | IgM | IgG3 |
| **Method to increase immunogenicity** | KLH conjugation | KLH conjugation | Adjuvant QUIL A | | KLH conjugation |
| **Route of injection** | IP | IP | SC | IV | IP |
| **Dose of Ab injection/ rat** | 250 μg | 500 μg | 500 μg | 500 μg | 500 μg |
| **Number of immunization** | 5 | 4 | 4 | 4 | 4 |
| **Time interval between immunizations** | 1 to 2 weeks | 1 week | 1 week | 1 week | 1 week |
| **Number of serum collection** | 6 | 5 | 5 | 5 | 5 |
| **Interval time between serum collection** | 1 to 2 weeks | 5-7 days | 5-7 days | 5-7 days | 5- 7 days |
| **Residual binding activity of therapeutic monoclonal antibodies** | HB1 (IgM)<br>40.9 % | HB1 (IgM)<br>43.93% | NA | NA | HB2 (IgG3)<br>71.97% |
| **Interval between the last immunization and the inflammation induction** | 5 days | 5 days | 5 days | 5 days | 5 days |
| **Length of time for inflammation measurement** | 7.5 hrs | 7.5 hrs | 6.5 hrs | 7.5 hrs | 7.5 hrs |
| **Number of inflammation measurements** | 8 | 8 | 7 | 8 | 8 |

Abbreviations: NA, not applicable.

**Table 6. Schedule of serum collection, immunization and inflammation induction for the first animal experiment**

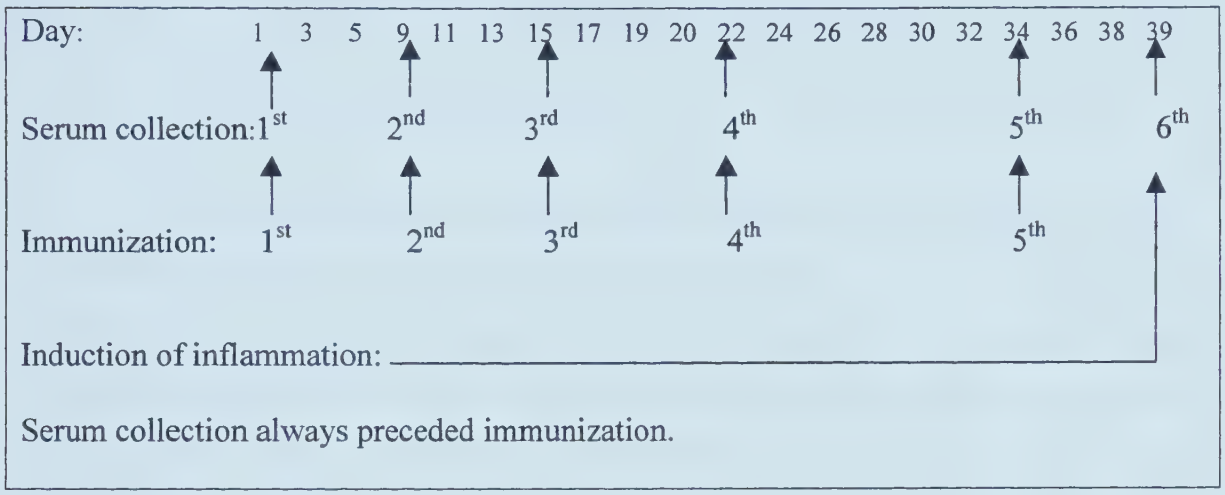
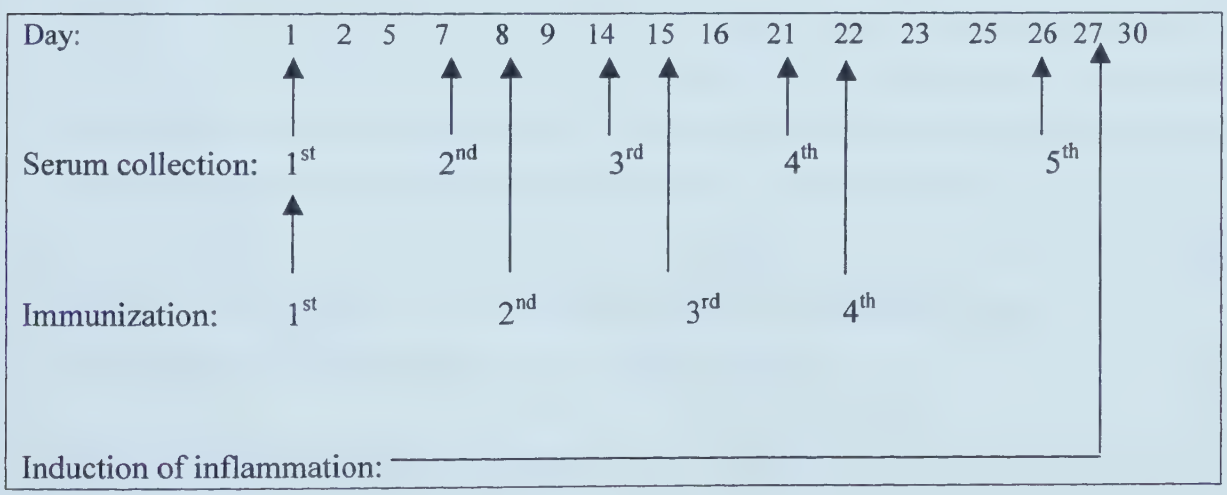



**Table 7. Schedule of serum collection, immunization and inflammation induction for the second, third, fourth and fifth animal experiments**



immersing the paw up to the tibio-tarsae articulation in a cylinder filled with water, and the fluid volume replaced by immersed paw was measured before and after carrageenan injection at desired time intervals as indicated in table 8.

### *2.8.5. Measurement of humoral immune responses*

#### *2.8.5.1. Measurement of RAMA response*

For the measurement of humoral RAMA (Rat anti-mouse antibody) response microtiter strips were coated with 100 µl of PBS containing 2.5 µg/ ml of HB1 mAb or HB2 mAb and incubated overnight at 4 °C. The coating solution was discarded and 150 µl of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS) was added to each well in order to block the non-specific binding sites on the wells. After incubation for 1 hour at room temperature the plates were washed three times with PBST. A 100 µl of binding buffer (1% BSA, 0.02% thimerosal in PBS) containing individual rat serum at 1/50 dilution was added to each well. All wells were incubated for 1 hr at room temperature, and followed by three washings with PBST. Then a 100 µl of binding buffer containing monoclonal anti-rat Kappa and Lambda light chain Ab-peroxidase conjugate was added to each well at a v/v dilution of 1/40,000, incubated for 1 hour, and followed by three washings with PBST. The activity of bound mAbs was determined by adding a 100 µl of ABTS (Peroxidase solution B and ABTS peroxidase substrate, 1:1 v/v dilution) to each well. The optical density was measured at dual wavelength, 405 and 492 using an ELISA reader (Titertek multiskan Plus MK II). The method used for the measurement of RAMA response is illustrated in figures 4.

#### *2.8.5.2. Measurement of Ab3 response*

For the measurement of humoral Ab3 response, the same procedure was used as in RAMA measurement except that the microtiter strips were coated with 100µl of PBS containing 65 U/ml of CA19-9 antigen. The method used for the measurement of Ab3 humoral response is illustrated in figure 5.

**Table 8. Schedule of inflammation measurements in each animal experiment**

| Experiment | Time in hours at which inflammation measurement was taken from the time of caragennan injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1.4 | 1.5 | 2.5 | 3.5 | 4.5 | 5.5 | 6.5 | 7.5 |
| Experiment 1 | x | x | | x | x | x | x | x | x |
| Experiment 2 | x | | x | x | x | x | x | x | x |
| Experiment 3 | x | | x | x | x | x | x | x | |
| Experiment 4 | x | | x | x | x | x | x | x | x |
| Experiment 5 | x | | x | x | x | x | x | x | x |

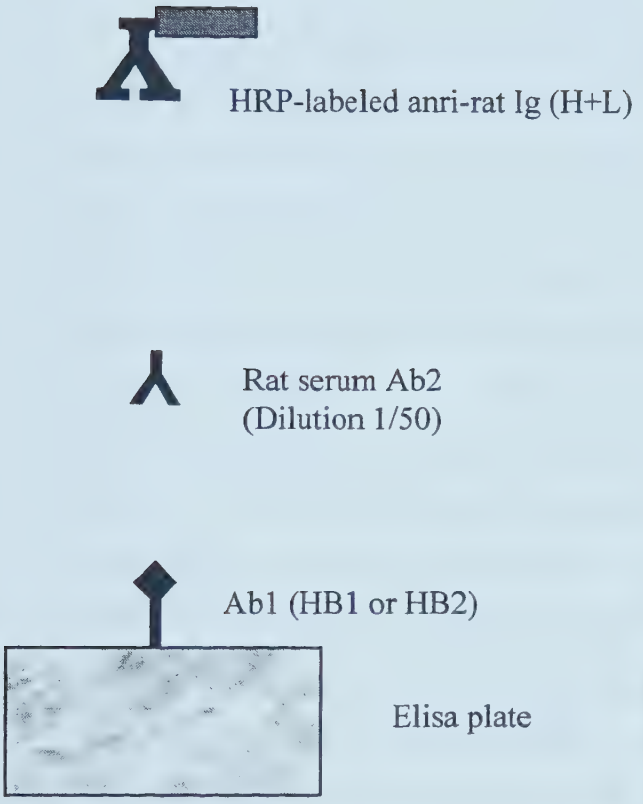



**Figure 4.** Illustration of Elisa for detection of RAMA

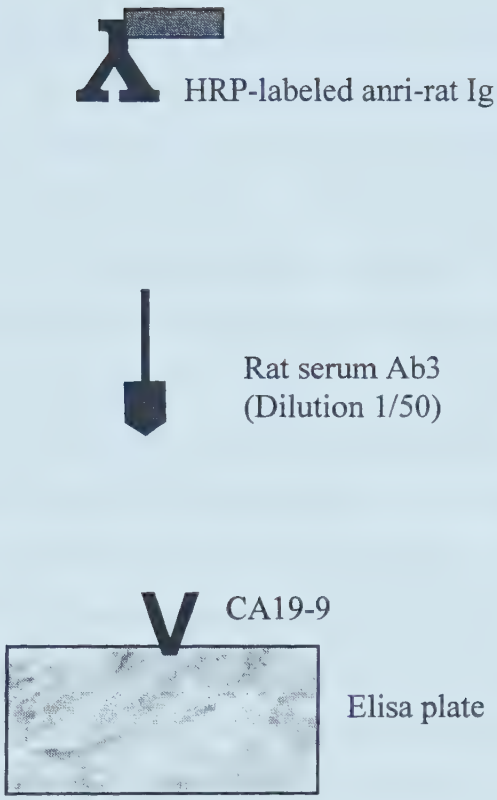



**Figure 5.** Illustration of Elisa for detection of Ab3

### *2.8.5.3. Competitive assays for the specific detection of idiotypic immune response*

#### *2.8.5.3.1. Competitive assay between RAMA positive rat serum and CA 19-9 antigen in the binding to HB1 mAb.*

Microtiter strips were coated with 100 µl of PBS containing either 2.5 µg/ml of HB1 mAb and incubated overnight at 4 °C. The coating solution was discarded and 150 µl of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS) was added to each well in order to block the non-specific binding sites on the wells. After one hour of incubation at room temperature the plates were washed three times with PBST. One set of wells was incubated with a 100 µl of binding buffer containing RAMA positive rat serum at a dilution of 1/50. Another set of wells was incubated with 100 µl of binding buffer containing RAMA positive rat serum at a dilution of 1/50 mixed with of 65 U/ml of CA19-9. Rat serum was collected from rats treated with either HB1 mAb or control F5 mAb. All wells were incubated for 1 hr at room temperature, followed by three washings with PBST. Then a 100 µl of binding buffer containing monoclonal anti-rat Kappa and Lambda light chain Ab-peroxidase conjugate was added to each well at a v/v dilution of 1/40,000, incubated for 1 hour, and followed by three washings with PBST. The activity of bound mAbs was determined by adding 100 µl of ABTS (Peroxidase solution B and ABTS peroxidase substrate, 1:1 v/v dilution) to each well. The optical density was measured at dual wavelength, 405 and 492 nm.

#### *2.8.5.3.2. Competitive assay between $I^{125}$ labeled HB1 antibody and RAMA positive rat serum in the binding to CA 19-9 antigen*

RIA strips coated with 100 µl of PBS containing 65 U/ml of CA19-9 antigen were incubated overnight at 4 °C. After discarding the coating solution, the nonspecific binding sites were blocked with 150 µl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature with gentle shaking, and then washed three times with PBST (PBS, 0.1%Tween-20, pH 7.1). One set of wells was incubated with 0.33 µg/ml of $I^{125}$ labeled HB1 antibody in 100 µl of binding

buffer (1% BSA, 0.02% thimerosal in PBS). Another set of wells was incubated with the same Ab mixed with RAMA positive rat serum at a dilution of 1/50 and incubated at room temperature for 1 hour. Rat serum was collected from rats treated with either HB1 mAb or control F5 mAb. Then 50 µl of the supernatant was taken from each well and the radioactivity was measured (free fraction) gamma counter. The strips were again washed three times with PBST, broken into individual wells and the radioactivity of each well was measured (bound fraction) by Gamma counter.

## *2.9. ELISA and RIA*

### *2.9.1. ELISA assay for determining the concentration or the binding activity of mAbs*

Microtiter strips were coated with 100 µl of PBS containing either 2.5 µg/ml of goat anti-mouse Ig (whole molecule), 75 U/ml of CA19-9 antigen or 2.5 µg/ml of polyacrylamide-SLe$^a$ and incubated overnight at 4 °C. The coating solution was discarded and 150 µl of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS) was added to each well in order to block the non-specific binding sites on the wells. After one hour of incubation at room temperature the plates were washed three times with PBST. A 100 µl of binding buffer containing mAbs was added in different dilutions. All wells were incubated for 1 hr at room temperature, followed by three washings with PBST. Then a 100 µl of binding buffer containing goat anti-mouse Ig (H+L) -HRP labeled was added to each well at a dilution of 1/5000 v/v, incubated for 1 hour, and followed by three washings with PBST. The activity of bound mAbs was determined by adding a 100 µl of ABTS (Peroxidase solution B and ABTS peroxidase substrate, 1:1 v/v dilution) to each well. The optical density was measured at dual wavelength, 405 and 492 nm.

### *2.9.2. RIA*

#### *2.9.2.1. Scatchard plot analysis for determining the affinity of radiolabeled HB1 mAb to CA19-9*

RIA strips coated with 100 µl of PBS containing 65 U/ml of CA19-9 antigen were incubated overnight at 4 °C. After discarding the coating solution, the nonspecific binding sites were blocked with 150 µl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature with gentle shaking, and then washed three times with PBST (PBS, 0.1%Tween-20, pH 7.1). Each strip was then incubated with a range of concentration from 0.005 to 10.5 µg/ml of $I^{125}$ labeled HB1 antibody in 100 µl of binding buffer (1% BSA, 0.02% thimerosal in PBS) at room temperature for 1 hour. Then 50 µl of the HB1 supernatant was taken from each well and the radioactivity was measured (free fraction) gamma counter.
The strips were again washed three times with PBST, broken into individual wells and the radioactivity of each well was measured (bound fraction) by Gamma counter.

#### ***2.9.2.2 Competitive assay for the determination of $SLe^a$ concentration required to inhibit the binding of $I^{125}$ labeled HB1 Ab to CA19-9***

RIA strips coated with 50 µl of PBS containing a 65 U/ml of tumor antigen CA19-9 were incubated overnight at 4 °C. After discarding the coating solution, the non-specific binding sites were blocked with 150 µl/well of blocking buffer (2% sucrose, 2% BSA, 0.06% thimerosal in PBS). The wells were incubated for 1 hour at room temperature, and then washed with PBST three times. Dilutions of $SLe^a$ antigen were made serially in binding buffer (1% BSA, 0.02% thimerosal in PBS) from 0 to 100 µg / ml and mixed with iodinated HB1 mAb. A nonsaturating concentration (0.33 µg/ml) of iodinated HB1 mAb was chosen for this experiment in order to obtain a final cpm concentration of approximately 40,000 cpm/well. A 100 µl of each dilution was added to different wells and allowed to incubate for 1.5 hrs. After that the supernatant was

discarded, the strips were washed with PBST three times and broken down to individual wells and the radioactivity was measured (bound fraction).

## *2.10. In vitro inhibition of leukocyte rolling on microchips by rolling assay*

To investigate the rolling and adhesion of cells, a 10 cm x 10 cm glass wafer with a series of simple "Y" mixer flow manifolds was used in this assay. The "Y" mixer flow manifold consisted of 50 microns in depth and 300 microns in width channels with a flow region downstream. These channels are structured in similar way to capillaries and therefore, the use of such channels allows the observation of cell rolling and adhesion in vitro that mimics the in vivo cell rolling and adhesion events (Li and Harrison, 1997). The microchip was secured into an aluminum microchip holder, and the microchip reservoirs were glued onto the microchip with 5-minute epoxy resin. The channels were conditioned with concentrated nitric acid ($HNO_3$), 2 M of sulfuric acid ($H_2SO_4$) and 1M of sodium hydroxide (NaOH) (10 minutes each) before and after each experiment. The channels were then coated with 20 μg/ml of Recombinant human E-selectin or P-selectin for 2 hours at 37 °C to mimic the surface of a vessel wall endothelial lining. A microsyringe pump that operates as a negative pressure source was used to drive the cells through the channels at a flow rate of 2.5 μl/min for the 50 micron deep device. This flow rate ensures a high enough shear rate (800 $s^{-1}$) to mimic blood flow in a vascular capillary. The channels were monitored using a Reichert Microstar (25X 280 x 220 μm field of view) and JVC color CCD camera mounted on the microscope (figure 40A). The experiments were recorded onto tapes with a JV VHS recorder. A 1x $10^7$ cells/ml solution of human PMNL or lymphocytes in RPMI, 5% FCS were pumped to the channel through an injection route and allowed to run at an optimum flow rate for the purpose of achieving the rolling step. Since each cannel has two sides, one side of each channel was used to test the agent under study and the other side of the channel was used as a control for cell rolling only without the addition of any agents (figure 40B). The cell suspensions added to different sides of the channel were regulated by a luminal fluidic flow that allowed these cell suspensions to continue running at different sides of the channel and prevented cell diffusion from one side of the channel to the other. The following samples

were tested at a volume of 10 μl and at a dilution of 1:10 v/v in RPMI and 5% FCS: fresh human serum (negative control), fresh rat serum, HB1 rat serum; and F5 rat serum. 10 μl of 100 μg/ml of Anti-E-selectin was used as positive control for E-selectin coated channels. Serum of rats immunized with HB1 mAb in the third experiment was obtained from rats who showed a high RAMA response (rat # 2, 4, 5, 6, 7 and 8 collectively). Serum of rats immunized with F5 mAb in the third animal experiment was obtained from rats who showed a high RAMA response (rat # 4, 6, and 7 collectively). Once the rolling step has been achieved, 10 μl of each of the above mentioned samples were added to different channels. The pump sucked it into the channel to start the inhibition step. The images of cell rolling and inhibition steps were captured at different times and the number of cells was counted in both sides of the channel. Non-adherent cells appear as streaks on the video image. The cell counts were conducted on a PC computer installed with a perception video board and image Pro Plus software.

The same samples were also tested on P-selectin coated channels under the same conditions as with E-selectin coated channels. For P-selectin coated channels, anti-P-selectin Ab was used as a positive control.

## *2.11. Statistics*

Statistics was done by ANNOVA ONE WAY (Tukey test), T TEST and linear regression.

# CHAPTER 3. RESULTS

## *3.1. Therapeutic monoclonal antibodies*

The therapeutic mAbs, HB1 and HB2, were kindly provided by AltaRex Corp. Those mAbs were produced by hybridoma fusion from mice immunized with the tumor antigen CA19-9. HB1 and HB2 mAbs were used in various animal experiments along with isotype matched control mAbs. The name, isotype and specificity of all Abs used in this study are indicated in table 4.

### *3.1.1. Purification of mAbs*

MAbs were purified from ascetic fluid using either chromatographic or precipitation methods as indicated in table 4.

The Mannan Binding Protein (MBP) is an affinity purification method for IgM Ab based upon the interaction of the carbohydrate moieties of IgM heavy chains with the mannan binding protein molecule (Nevens et al., 1992). This method was used for the purification of HB1 and F5 IgM mAbs.

The protein A affinity chromatography is a purification method for IgG and is based upon the interaction of the Fc portion of IgG with immobilized protein A. This method was used for the purification of Flopc-21 $IgG_3$ mAbs.

Euglobulin precipitation is a non-chromatographic method for the purification of murine $IgG_3$ and IgM Abs that takes advantage of their euglobulin properties.

High yield of purification was obtained with all the methods used, as indicated in table 4. MBP affinity chromatography was performed 8 times for HB1 antibody and 6 times for F5 antibody purification and therefore, an average yield of purification for each of them was shown in table 4. Each one of the other purification methods was used only once. Degree of purity analyzed by SDS PAGE (figure 6) showed lower degree of purity for euglobulin precipitation method. Extra bands appear along with the expected bands of 7C2C5C12 (IgM) or HB2 ($IgG_3$). The molecular weight of these extra bands suggests

**Table 4. Control and therapeutic antibodies**

| Antibody | Isotype | directed against | tested as | Mode of purification | Concentration in ascetic fluid | Yield of purification |
|---|---|---|---|---|---|---|
| HB1 | IgM | CA19-9 antigen | Therapeutic | Mannan binding protein affinity chromatography | 0.79 mg/ml | 91.35% |
| F5 | IgM | Human prostate cancer antigen | Control | Mannan binding protein affinity chromatography | 1.36 mg/ml | 81.4% |
| 7C2C5C12 | IgM | Trichinella spiralis | Control | Euglobulin precipitation method | 2.3 mg/ml | 78% |
| HB2 | $IgG_3$ | CA19-9 antigen | Therapeutic | Euglobulin precipitation method | 0.7 mg/ml | 80.7% |
| Flopc-21 | $IgG_3$ | Unknown | Control | Protein A affinity chromatography | 0.78 mg/ml | 84.6% |

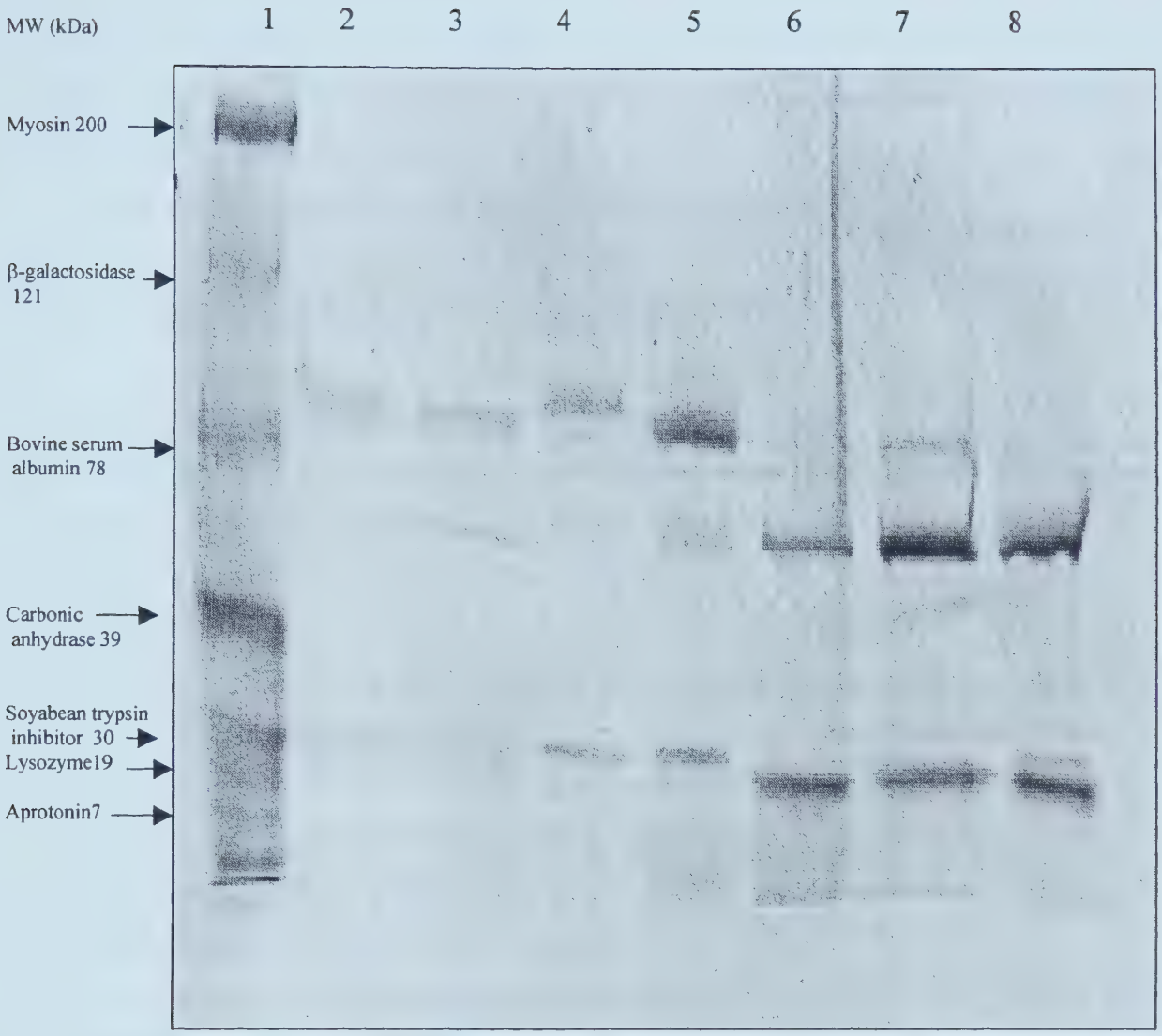



**Figure 6.** SDS-polyacrylamide Gel Electrophoresis of purified monoclonal antibodies performed under reducing conditions with 10 % acrylamide Tris HCl ready gel. (1) Kaleidoscope prestained standards (molecular weight marker); (2) F5 (IgM); (3) 7C2C5C12 (IgM); (4) HB1 (IgM); (5) HB1 (IgM); (6) Flopc-21, ($IgG_3$);(7) HB2 ($IgG_3$); and (8) HB2 ($IgG_3$) antibodies. Bands in lanes 2 and 4 correspond to antibodies that were purified by Mannan binding protein affinity chromatography, lanes 3 and 7 by euglobulin precipitation method and lane 6 by protein A affinity chromatography. Bands in lanes 5 and 8 correspond to old samples of purified monoclonal antibodies.

that the contaminating proteins correspond to either IgG or IgM respectively produced by the ascites bearing mice. Although euglobulin purification method resulted in slightly lower yield of purification and lower degree of purity compared to affinity methods, this method is considerably less time consuming and less expensive.

### *3.1.2. Specificity of the therapeutic monoclonal antibodies*

The binding of HB1 and HB2 mAbs to CA19-9 antigen was studied by direct ELISA (figure 7). In this experiment a range of mAb concentration from 0 to 0.64 μg/ml was used. For both HB1 and HB2 Abs even at a concentration of 10 ng/ml maximum binding to CA19-9 was observed which is likely to be due to the presence of multiple epitopes within the CA19-9 antigen.

The two control mAbs F5 and 7C2C5C12 did not bind to CA19-9 antigen as expected. Surprisingly, the control mAb Flopc-21 that was used in the same concentration range bound to CA19-9 with an observed dose effect. In order to determine more specifically the epitope recognized by HB1 and HB2 we used the carbohydrate antigen $SLe^a$ conjugated to polyacrylamide in order to carry a direct binding assay with polyacrylamide-$SLe^a$ antigen immobilized on ELISA plates.

This experiment allowed us to demonstrate that both HB1 and HB2 mAbs recognized the carbohydrate epitope $SLe^a$ as illustrated in figure 8. In addition, with this experiment we confirmed that Flopc-21 mAb is directed against a different epitope determinant than HB2 and HB1 and does not interact with $SLe^a$ carbohydrate Ag as shown in figure 8.

The specificity of HB1 mAb for $SLe^a$ was also studied by competitive inhibition assay. As shown in figure 9, $SLe^a$ antigen only at high concentrations, 50 μg /ml and 100 μg/ml, was able by competitive inhibition to reduce the binding of HB1 to CA19-9 antigen by 35% and 60% respectively. The requirement for such a high concentration of $SLe^a$ to inhibit the binding of HB1 mAb to CA19-9 could be due to the fact that HB1 Ab is an IgM and therefore has 10 binding sites for $SLe^a$ molecule. In addition, the affinity of HB1 Ab for $SLe^a$ epitope carried by CA19-9 is likely to be higher than the affinity of HB1 Ab for soluble $SLe^a$. Such difference in affinity may be due to a more stabilized three-

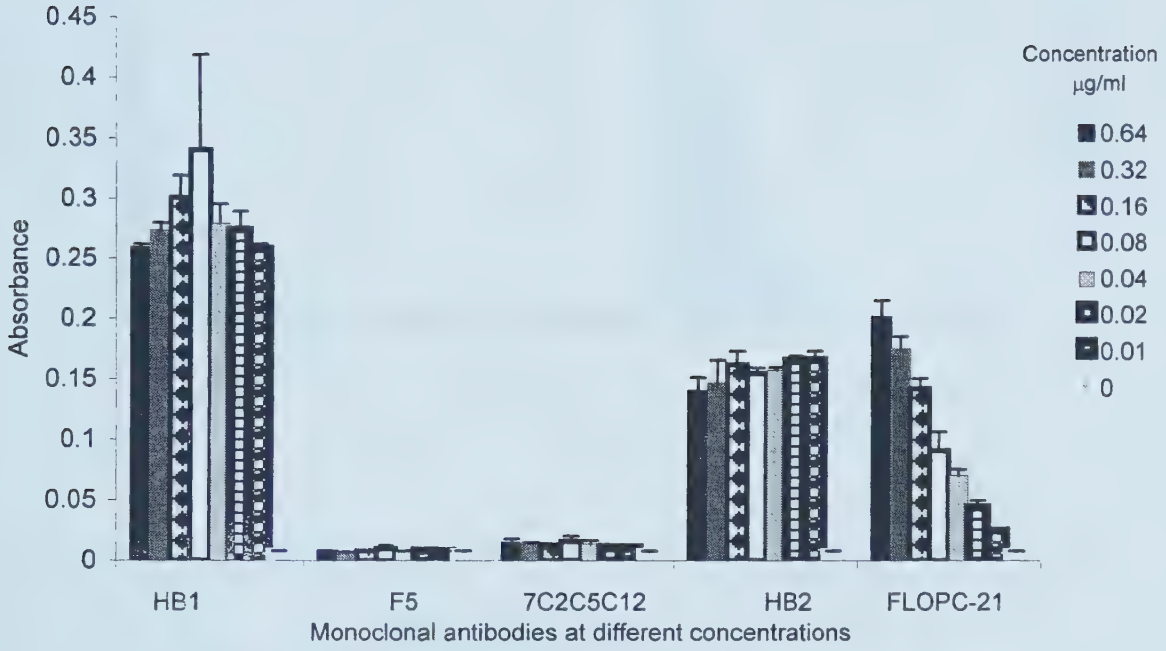



**Figure 7.** Binding of various murine monoclonal antibodies to tumor antigen CA19-9. The assay was performed by ELISA using CA19-9 coated plates as described in materials and methods. The tested mAbs include control mAbs 7C2C5C12 (IgM), F5 (IgM) and Flopc-21(IgG3); and therapeutic mAbs HB1 (IgM) and HB2 (IgG3).

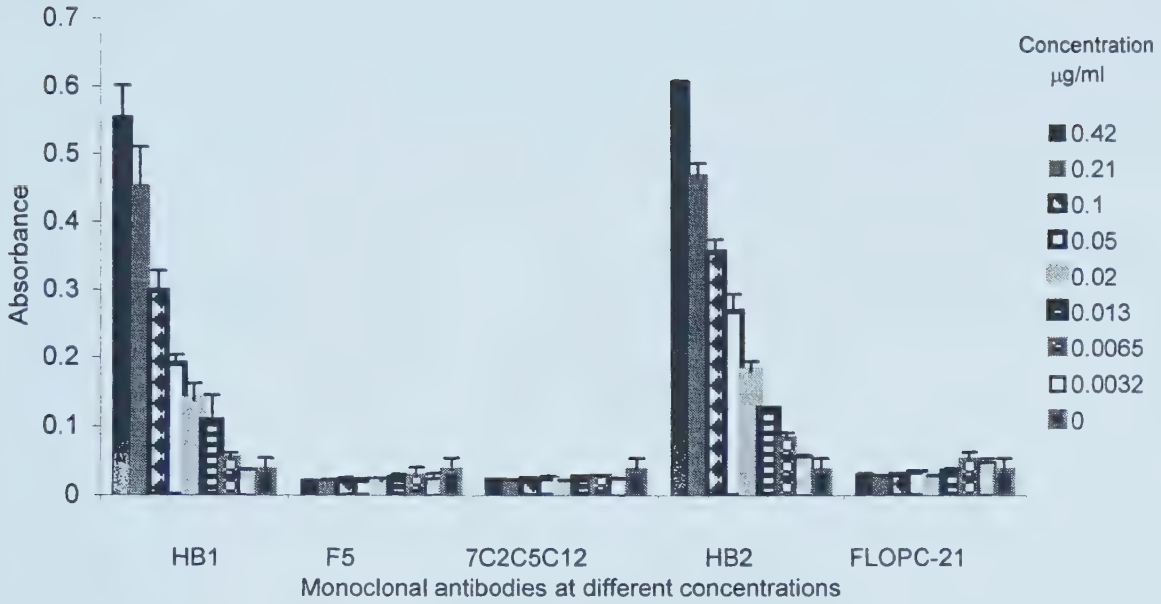



**Figure 8.** Binding of various murine monoclonal antibodies to sialyl Lewis A oligosaccharide. The assay was performed by ELISA using polyacrylamide-Sialyl Lewis A coated plates as described in materials and methods. The tested mAbs include control mAbs 7C2C5C12 (IgM), F5 (IgM) and Flopc-21 (IgG3); and therapeutic mAbs HB1 (IgM) and HB2 (IgG3).

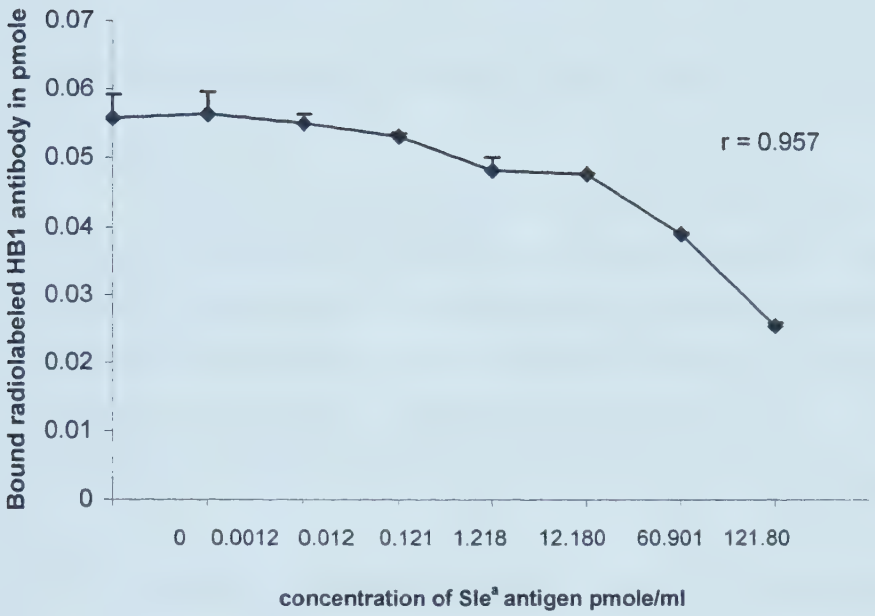



**Figure 9.** The inhibitory effect of sialyl Lewis A antigen on the binding of iodine 125 radiolabeled HB1 mAb to immobilized CA19-9. RIA plates were coated with CA19-9 at a concentration of 65 U/ml and incubated with iodinated HB1 mAb at a concentration of 0.33 μg/ml and increasing concentration of SLe A antigen (0-100 μg/ml).The concentration of iodinated HB1 mAb used in this assay (0.33 μg/ml) was shown to be nonsaturating.

dimensional configuration of the $SLe^a$ carried by CA19-9 and/or to a longer sequence recognized by HB1 within CA19-9 molecule.

The affinity of HB1 mAb to CA19-9 antigen was determined by scatchard analysis using $^{125}I$ radiolabeled HB1 mAb. As shown in figure 10, the Kd value from this experiment was calculated to be equal to 0.94 nM.

### *3.1.3. Binding activity of therapeutic mAbs after conjugation to KLH*

The mAbs were conjugated to KLH in order to enhance their immunogenicity. Chemical conjugation is usually very efficient but is likely to decrease the binding activity of the mAbs. For each conjugation, we therefore determined the remaining binding activity of the KLH-mAbs. This was done by direct ELISA using CA19-9 coated plates. The absorbance of the conjugated Abs with the unconjugated ones were compared and used to calculate the residual binding activity of the conjugated mAbs (table 5).

## *3.2. Evaluation of the therapeutic efficacy of the tested mAbs in vivo*

Subsequent to the treatment of rats with the therapeutic mAb, control mAb or PBS, inflammation was induced in the rat's right hind paw by carrageenan injection. Two methods were used to measure paw edema. Caliper method was used to measure paw thickness and water displacement method for paw volume. The therapeutic efficacy of the tested mAb was evaluated by comparing the extent of inflammatory response in different rat groups.

The induction of an anti-idiotypic immune response was tested in each rat group. The immune response induced against the injected therapeutic mAb was measured by two different ELISAs illustrated in figure 4 and 5. The assay described in figure 4 measured the RAMA (rat anti-mouse antibodies) immune response which includes the anti-idiotypic immune response (Ab2), the anti-isotypic and anti-allotypic immune responses. The assay described in figure 5 measured the anti-anti-idiotypic immune response (Ab3).

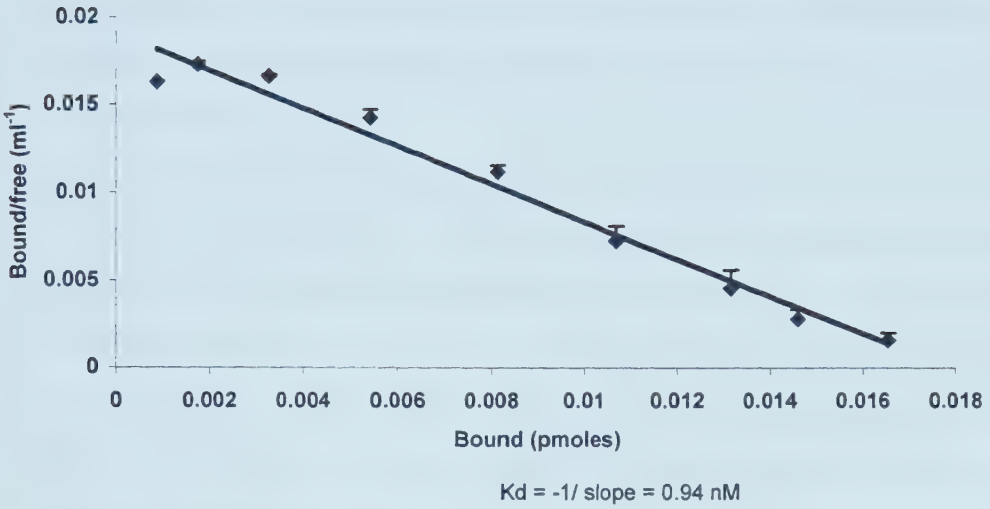



**Figure 10.** Scatchard analysis of the binding of iodinated HB1 mAb to immobilized CA19-9 antigen. RIA strips were coated with CA19-9 at a concentration of 65 U/ml and radiolabeled HB1 antibody was added to strips at different dilutions from 0.005 to 10.5 μg/ml. The affinity of iodinated HB1 antibody to CA19-9 antigen was determined by measuring the radioactivity of free and bound fractions in individual wells by Gamma counter.

For the specific detection of idiotypic immune response, different competitive assays were carried out as described in section 2.8.5. The assay described in section 2.8.5.3.1 showed small inhibition of RAMA response but the results (not shown) were not consistant. The assay described in section 2.8.5.3.2. showed inhibition of RAMA with both, control F5 and therapeutic HB1 mAbs rat serum. Therefore, this assay was not a specific assay.

Four independent experiments with HB1 and one experiment with HB2 were performed as described in table 5. In the case of HB1, rats were immunized with mAbs by three different routes of injection, ip, sc and iv. QUIL A adjuvant was used for subcutaneous route of injection. Two different doses of mAbs were tested, 250 μg and 500 μg per animal. Two forms of mAbs were tested: unconjugated and KLH conjugated. In the case of HB2, rats were immunized with KLH conjugated mAbs by ip injection at a dose of 500 μg per animal.

### *3.2.1. Experiment 1*

Rats in experiment 1, have been injected five times with 250 μg/rat of either KLH-conjugated HB1 mAb, control KLH-conjugated F5 mAb, or have simply received PBS injection via ip route (table 6). RAMA and Ab3 responses were measured between each injection as indicated in table 6. Rats were subjected to acute inflammation in the right hind paw by carrageenan injection 5 days after the last injection as indicated in table 5. Paw edema as a characteristic sign of inflammation was quantitated at regular interval of time with a total of 8 measurements as indicated in table 8.

#### *3.2.1.1. Therapeutic effect*

For both, paw thickness and paw volume measurements of the inflammatory response results were analyzed in the same way. They were plotted as AUC % change in effect (area under the curve) and as % change in maximum effect. Percent change in effect means % change in inflammatory response in each rat from time zero to each consecutive time of inflammation measurement. AUC % change in effect (area under the

curve); means the sum of % changes in inflammatory response from time zero to each consecutive time of inflammation measurement for each rat. The average of AUC% change in effect is calculated per rat group. Percent change in maximum effect means the % change in inflammatory response from time zero to the time where maximum inflammation has occurred in each rat.

Paw thickness measurement results are illustrated as AUC % change in effect in figures 11A, 11B, & 11C; and as % change in maximum effect in figures 12A, 12B, &12C. Figures 11A, 11B & 11C represent the same data, illustrated in three different ways. The same applies for figures 12A, 12B, & 12C.

Paw volume measurement results are illustrated as AUC % change in effect in figures 13A, 13B, & 13C; and as % change in maximum effect in figures 14A, 14B, & 14C. Figures 13A, 13B, & 13C represent one graph, illustrated in three different ways. The same applies for figures 14A, 14B, & 14C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect, measured by caliper method (P= 0.631) or by water displacement method (P= 0.655) was not statistically significant.

#### *3.2.1.2. Immune response*

Ab3 was not detected in any of the rat groups tested (data not shown). The RAMA response was detected in only one rat in the group of rats treated with HB1 mAb. The results obtained for the measurement of the RAMA immune response are shown in figure 15A. PBS group did not receive any mAb injection and consequently had no RAMA response. The absorbance obtained by ELISA corresponds therefore to background level. F5 group has shown absorbance values above background level. These values correspond to anti-isotypic and/or anti-allotypic immune responses (the assay performed with HB1 coated plates can not measure the anti-idiotypic response specific for F5 antibody). HB1 group showed absorbance values above background level that corresponds to either anti-isotypic, anti-idiotypic or anti-allotypic immune response or a

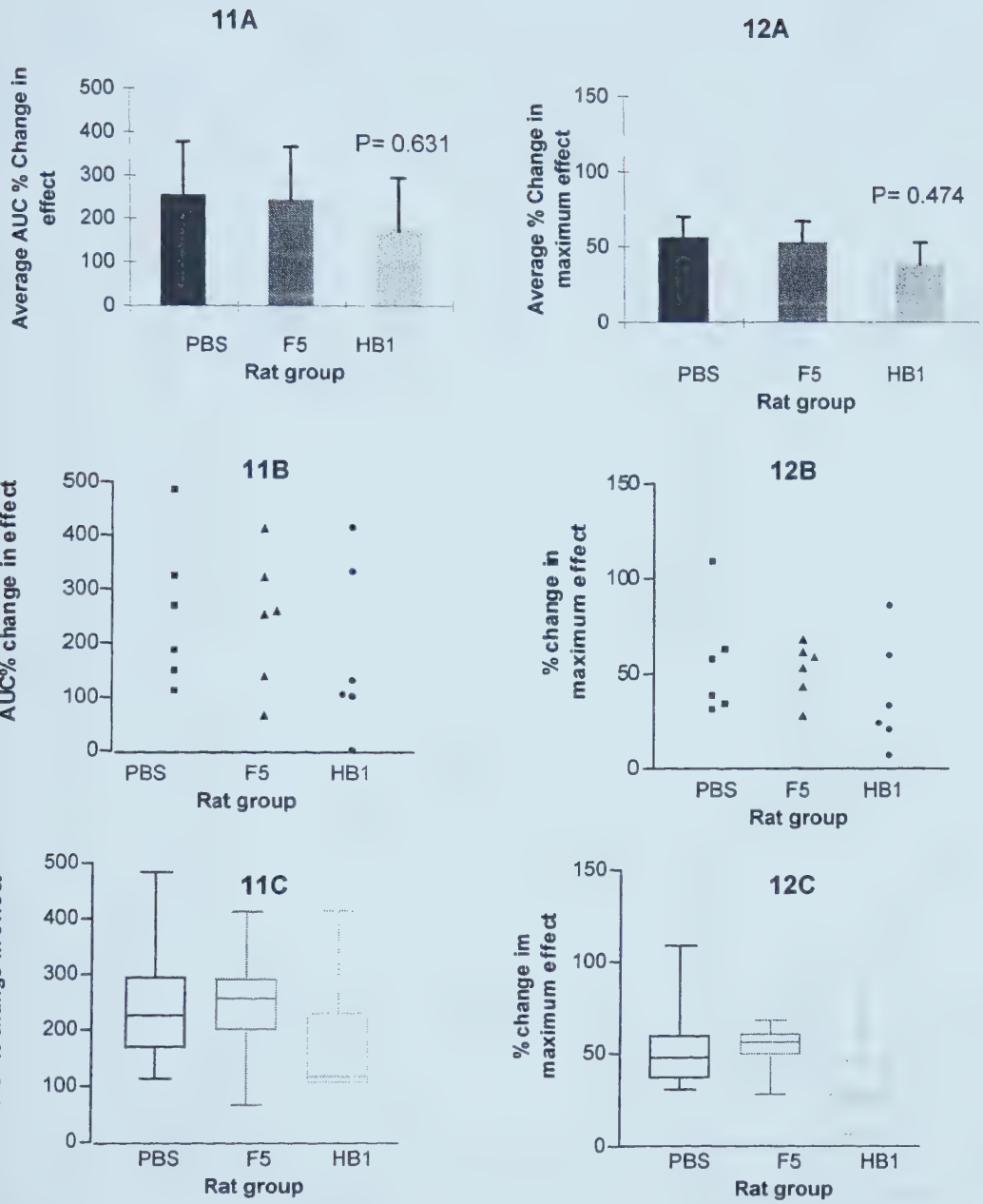



**Figure 11 and 12.** Comparison of the inflammatory response in different rat groups for the first experiment as measured by caliper method.

Rats in experiment 1, that have been previously immunized with either HB1 mAb, F5 control mAb, or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 11A, 11B & 11C, and as % change in maximum effect in figures 12A, 12B & 12C. The same results are plotted as bar graphs (A), column scatter (B), and whicker box (C). % change in effect means the % change in inflammatory response from time zero to each consecutive time of inflammation measurement. % change in maximum effect means the % change in inflammatory response from time zero to the time where maximum inflammation has occured. AUC % change in effect (area under the curve) means the sum of % change in effect of the inflammatory response from time zero to each consecutive time of inflammation measurement.

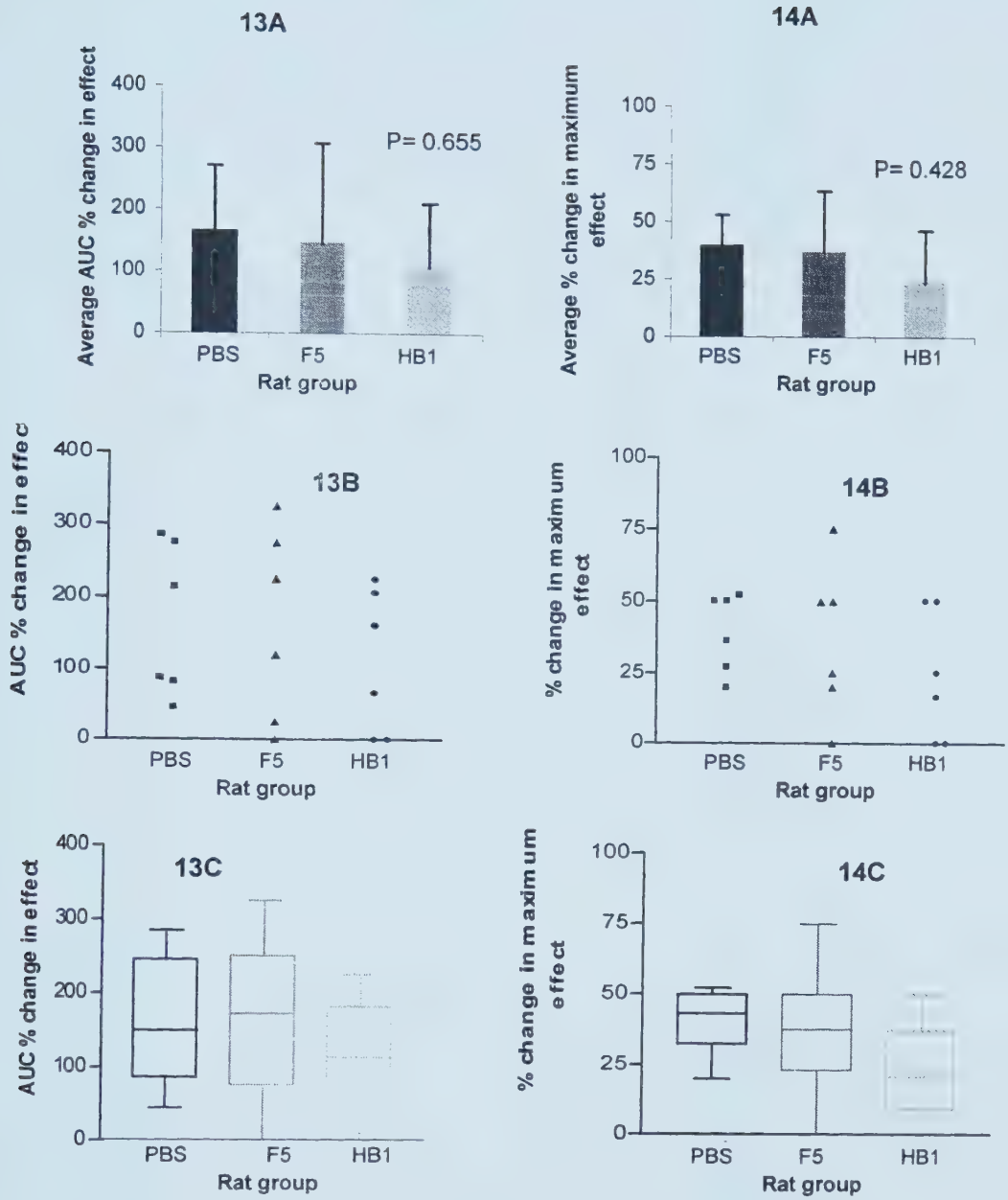



**Figure 13 and 14.** Comparison of the inflammatory response in different rat groups for the first experiment as measured by water displacement method.

Rats in experiment 1, that have been previously immunized with either HB1 mAb, F5 control mAb, or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw volume at regular interval of time, by water displacement method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 13A, 13B & 13C, and as % change in maximum effect in figures 14A, 14B & 14C. The same results are plotted as bar graphs (A), column scatter (B), and whicker box (C).

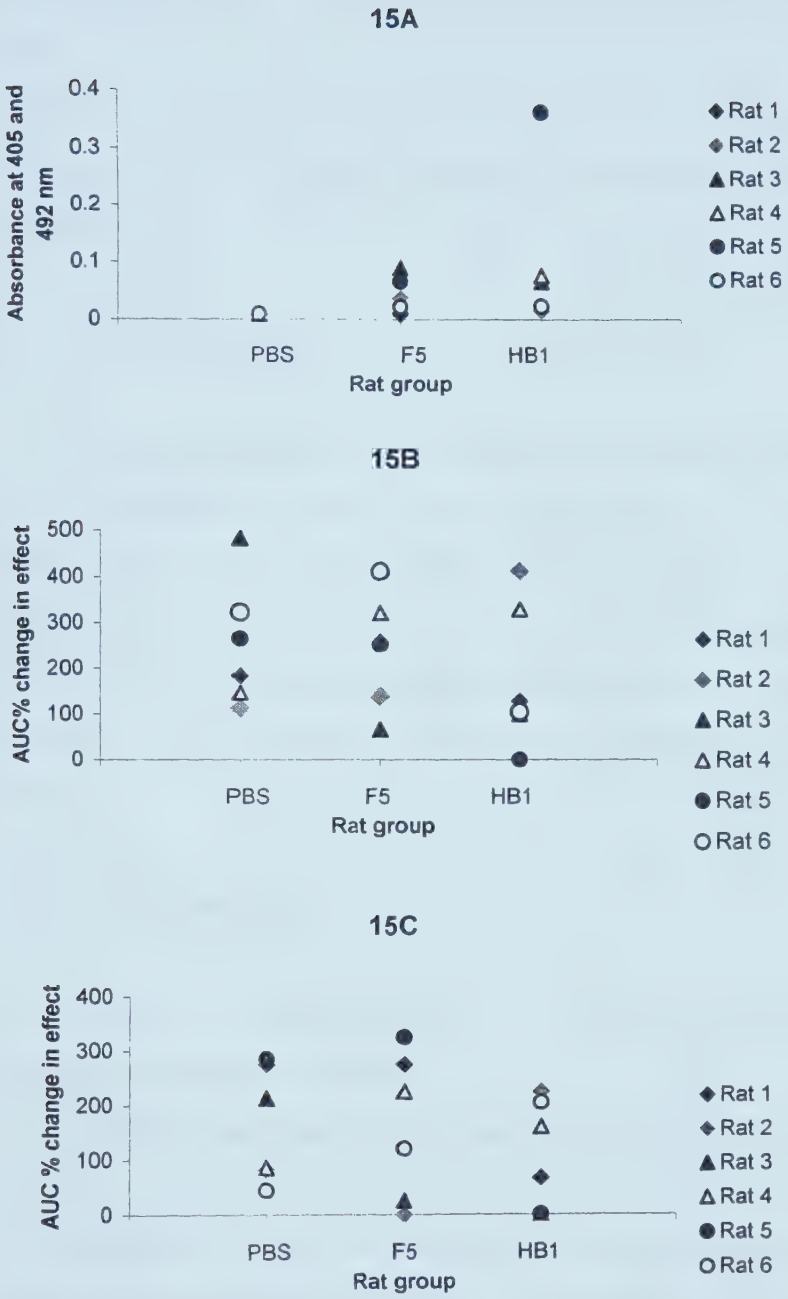



**Figure 15.** Comparison of the RAMA response with the inflammatory response in different rat groups for the first experiment.

Prior to inflammation induction serum was collected and tested for the presence of RAMA response by the method illustrated in figure 4. The results plotted as column scatter graph in figure 15A represents the extent of the RAMA response in different groups. Figures 15B and 15C representing the the extent of the inflammatory response measured by caliper method and water displacement method respectively have been shown and explained previously as figures 11B and 13B respectively. These two graphs are shown again for the convenience of comparing the extent of the inflammatory response with the level of the immune response in the three rat groups.

combination of them, among which only the idiotypic response will have the therapeutic effect.

It is worth noting that in the HB1 group, one rat (rat # 5) had a high RAMA response. Of particular interest, this particular rat did not develop any inflammatory response (figures 15B and 15C).

### *3.2.2. Experiment 2*

Rats in experiment 2, have been injected four times with 500 μg/rat of either KLH-conjugated HB1, control KLH-conjugated F5, control KLH-conjugated 7C2C5C12 mAbs, or have simply received PBS injection via ip route as described in table 5. RAMA and Ab3 responses were measured between each injection as indicated in table 7. Induction and measurement of inflammation were done by the same methods as in experiment 1. Paw edema was observed and measured at intervals of time indicated in table 8.

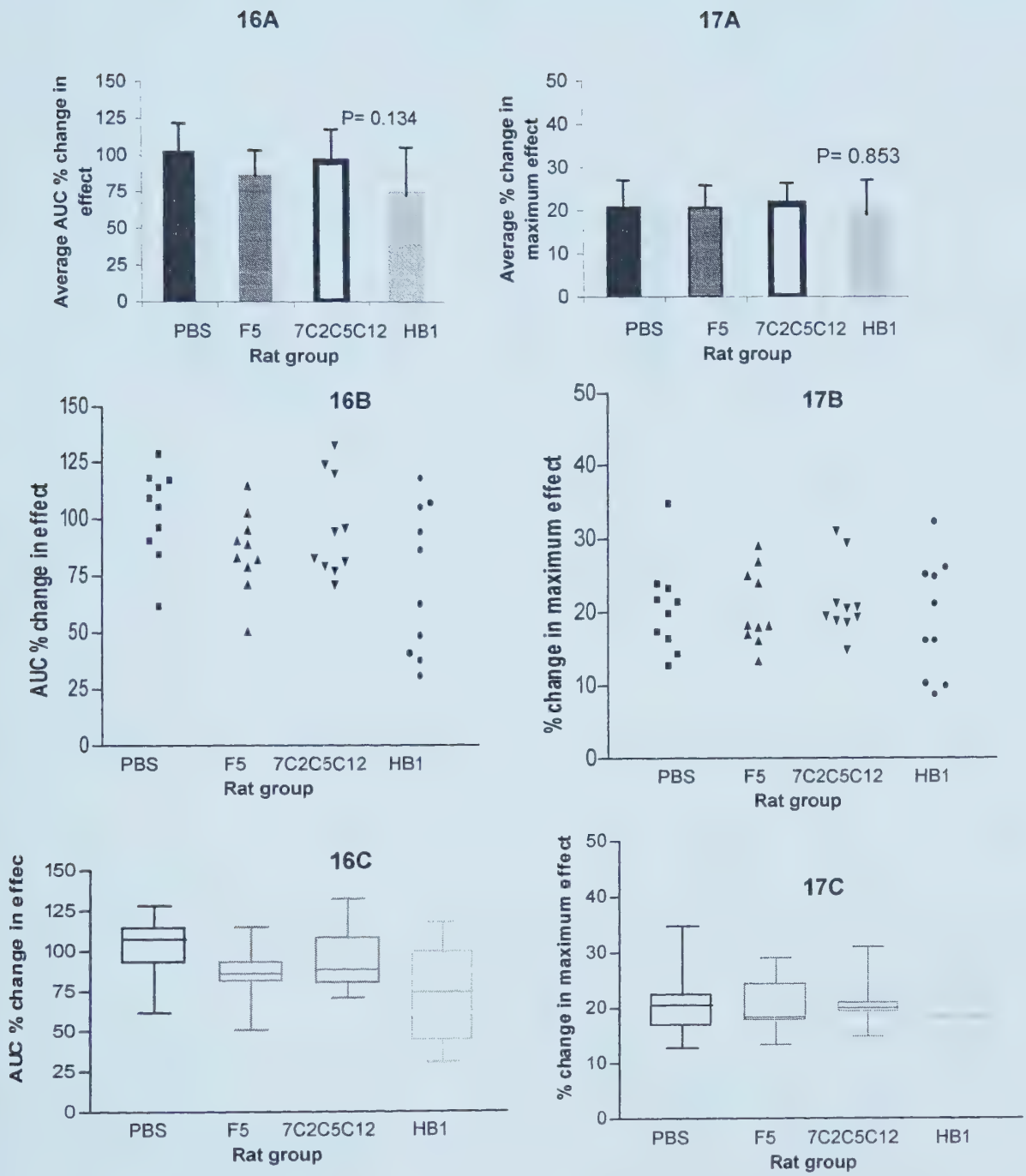
#### *3.2.2.1. Therapeutic effect*

Paw thickness and paw volume measurement results were analyzed and plotted in the same way as in experiment 1.

Paw thickness measurement results are illustrated as AUC % change in effect in figure 16A, 16B, & 16C; and as % change in maximum effect in figures 17A, 17B, & 17C. Figures 16A, 16B & 16C represent the same data, illustrated in three different ways. The same applies for figures 17A, 17B, & 17C.

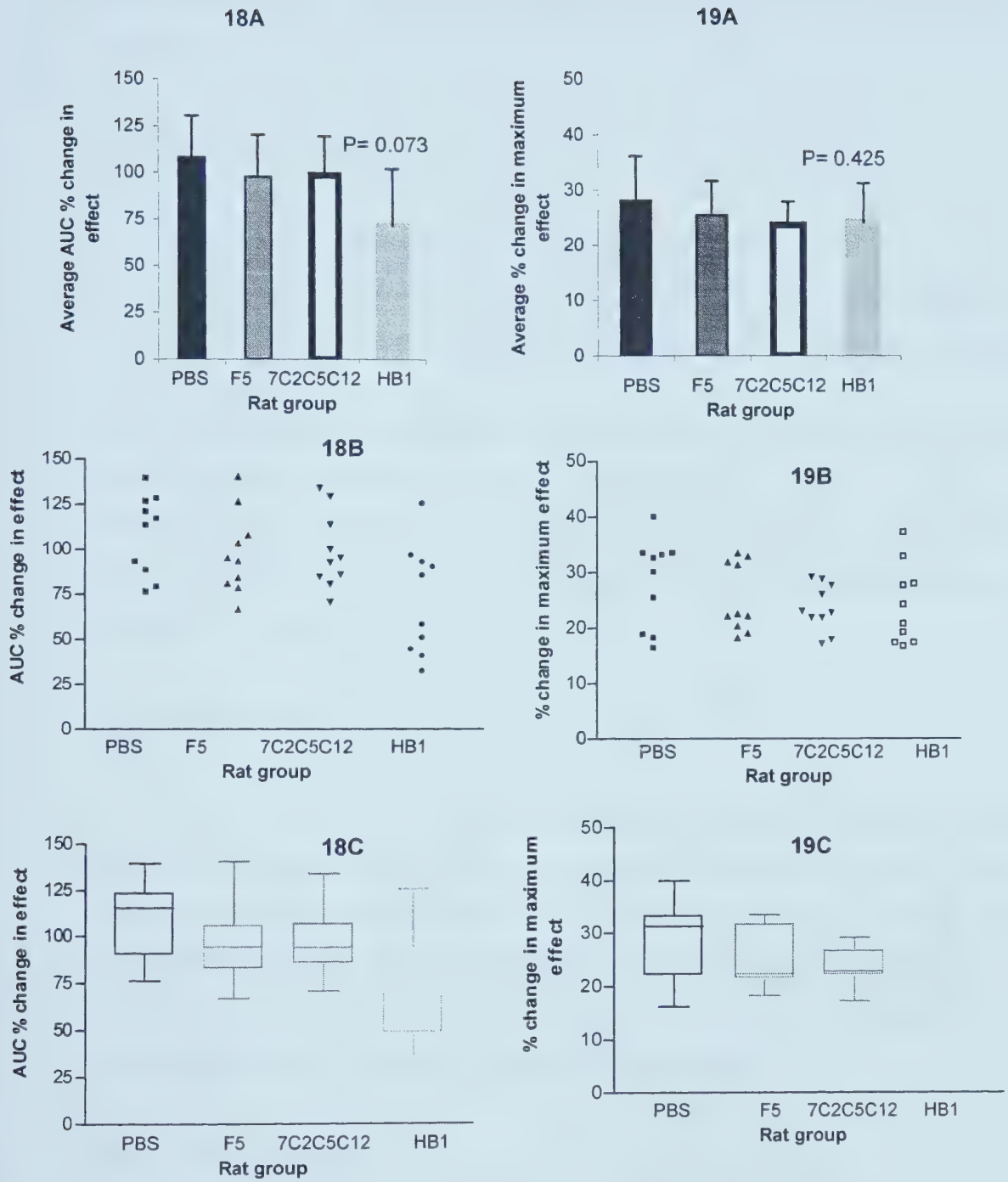
Paw volume measurement results are illustrated as AUC % change in effect in figures 18A, 18B, & 18C; and % change in maximum effect in figures 19A, 19B, & 19C. Figures 18A, 18B, & 18C represent one graph, illustrated in three different ways. The same applies for figures 19A, 19B, & 19C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect, measured by caliper




**Figures 16 and 17.** Comparison of the inflammatory response in different rat groups for the second experiment as measured by caliper method.

Rats in experiment 2, that have been previously immunized with either HB1 mAb, F5 control mAb, 7C2C5C12 control mAb or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection.The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 16A, 16B and 16C; and as % change in maximum effect in figures 17A, 17B and 17C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).




**Figures 18 and 19.** Comparison of the inflammatory response in different rat groups for the second experiment as measured by water displacement method.
Rats in experiment 2, that have been previously immunized with either HB1 mAb, F5 control mAb, 7C2C5C12 control mAb or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection.The extent of the inflammatory response was determined by measuring paw volme at regular interval of time, by water displacement method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 18A, 18B and 18C; and as % change in maximum effect in figures 19A, 19B and 19C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).

method (P= 0.134) or by water displacement methods (0.073) was not statistically significant.

#### *3.2.2.2. Immune response*

Ab3 was not detected in any of the rat groups tested.

A very low RAMA immune response was detected in the control groups (F5 and 7C2C5C12). HB1 group was considered the only group with a high RAMA immune response (figure 20A).

Despite the fact that the group treated with HB1 Ab had the highest immune response, we found no significant correlation between the RAMA response and the therapeutic efficacy as shown in figure 20. The correlation between the therapeutic efficacy and RAMA in HB1 group was not found to be significant at any time of the inflammation measurements (figures 21A-21H).

### *3.2.3. Experiment 3*

Rats in experiment 3, have been injected four times with 500 μg/rat of either HB1, control F5 mAbs mixed with QUIL A adjuvant, or have simply received PBS injection via sc route (table 5). RAMA and Ab3 responses were measured between each injection at regular intervals as indicated in table 7. Induction and measurement of inflammation was done by the same methods as in experiment 1. Paw edema was observed and measured at intervals of time indicated in table 8.

#### *3.2.3.1. Therapeutic effect*

Paw thickness and paw volume measurement results were analyzed and plotted in the same way as in experiment 1.

Paw thickness measurement results are illustrated as AUC % change in effect in figures 22A, 22B, & 22C; and as % change in maximum effect in figures 23A, 23B, &

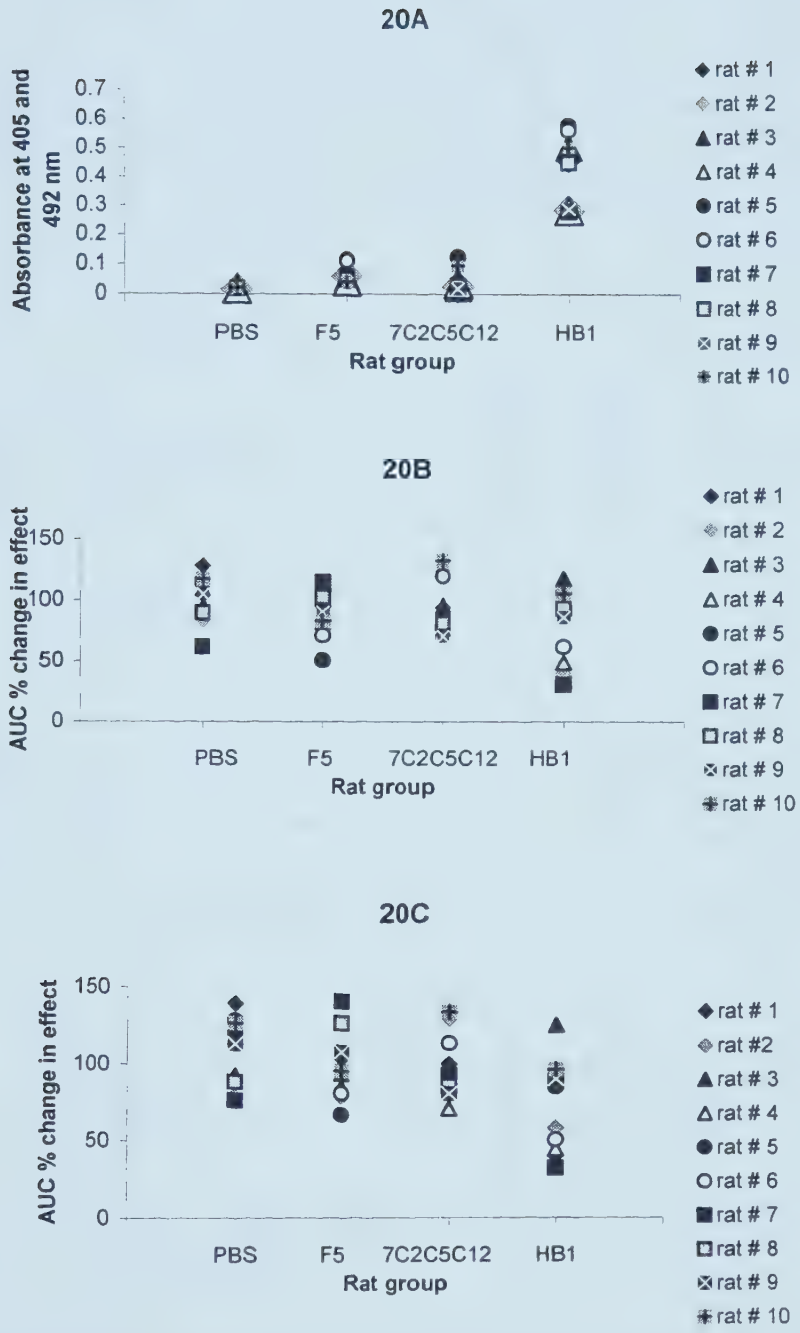



**Figure 20.** Comparison of the RAMA response with the inflammatory response in different rat groups for the second experiment.

Prior to inflammation induction serum was collected and tested for the presence of RAMA response by the method illustrated in figure 4. The results plotted as column scatter graphin figure 20A represents the extent of RAMA response in different rat groups . Figures 20B and 20C representing the extent of the inflammatory response measured by caliper and water displacement method respectively have been shown and explained previously as figures 16B and 18B respectively. These two graphs are shown again for the convenience of comparing the extent of the inflammatory response with the level of the immune response in the three rat groups.

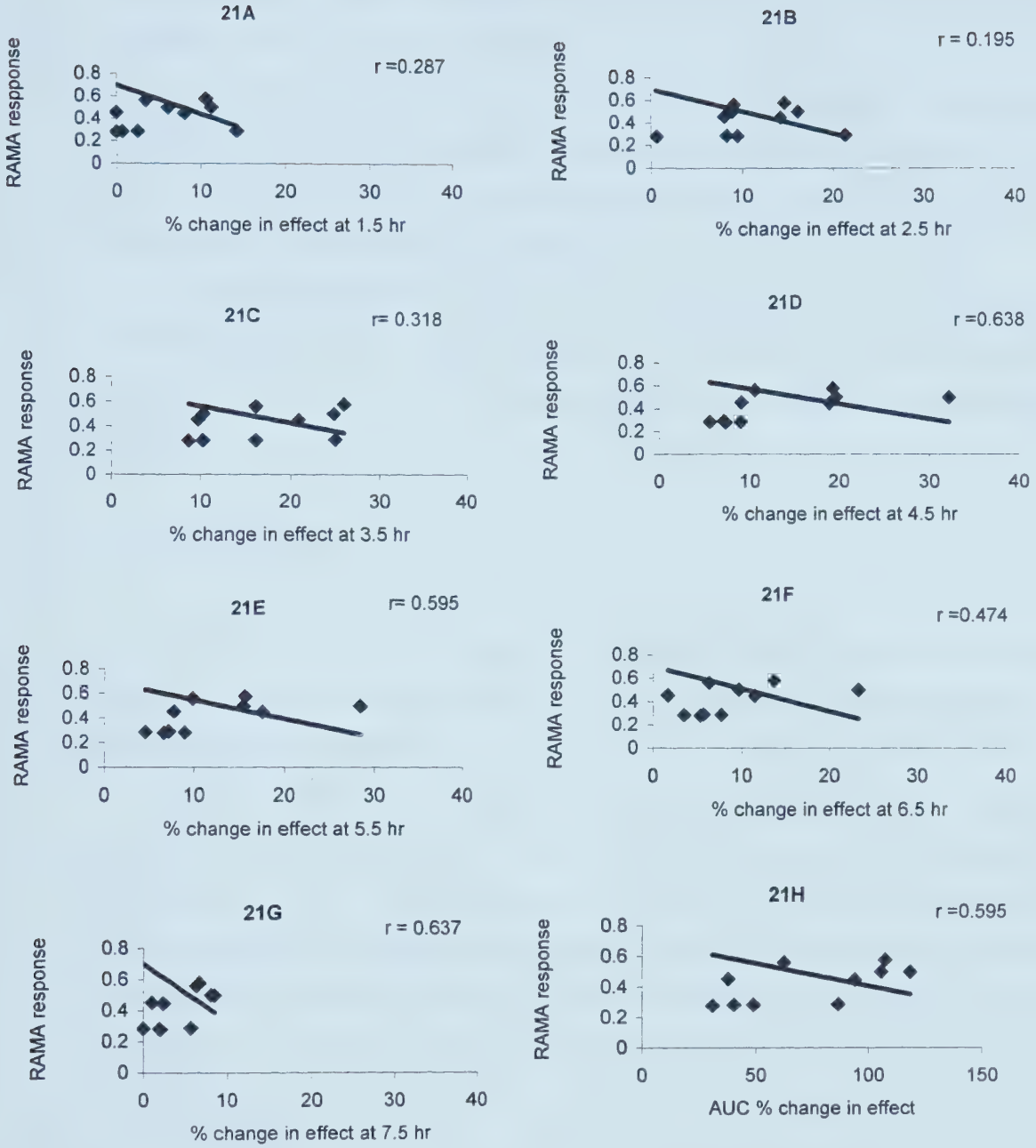



**Figure 21.** Correlation between RAMA response and the inflammatory response at different time measurements in the rat group treated with HB1 mAb in the second experiment.
Rat that have been previously immunized with HB1 mAb were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 8 measurements were performed. Serum was collected from the same group prior to inflammation induction and tested for the presence of RAMA response. The inflammatory response at different measurement time is plotted as % change in effect against the RAMA response. The inflammatory response was measured at 1.5 hr (A), 2.5 hr (B), 3.5 hr (C), 4.5 hr (D), 5.5 hr (E), 6.5 hr (F), 7.5 hr (G). The AUC % change in effect is plotted against RAMA response in graph (H)

23C. Figures 22A, 22B & 22C represent the same data, illustrated in three different ways. The same applies for figures 23A, 23B, & 23C.
Paw volume measurement results are illustrated as AUC % change in effect in figures 24A, 24B, & 24C; and as % change in maximum effect in figures 25A, 25B, & 25C. Figures 24A, 24B, & 24C represent one graph, illustrated in three different ways. The same applies for figures 25A, 25B, & 25C.

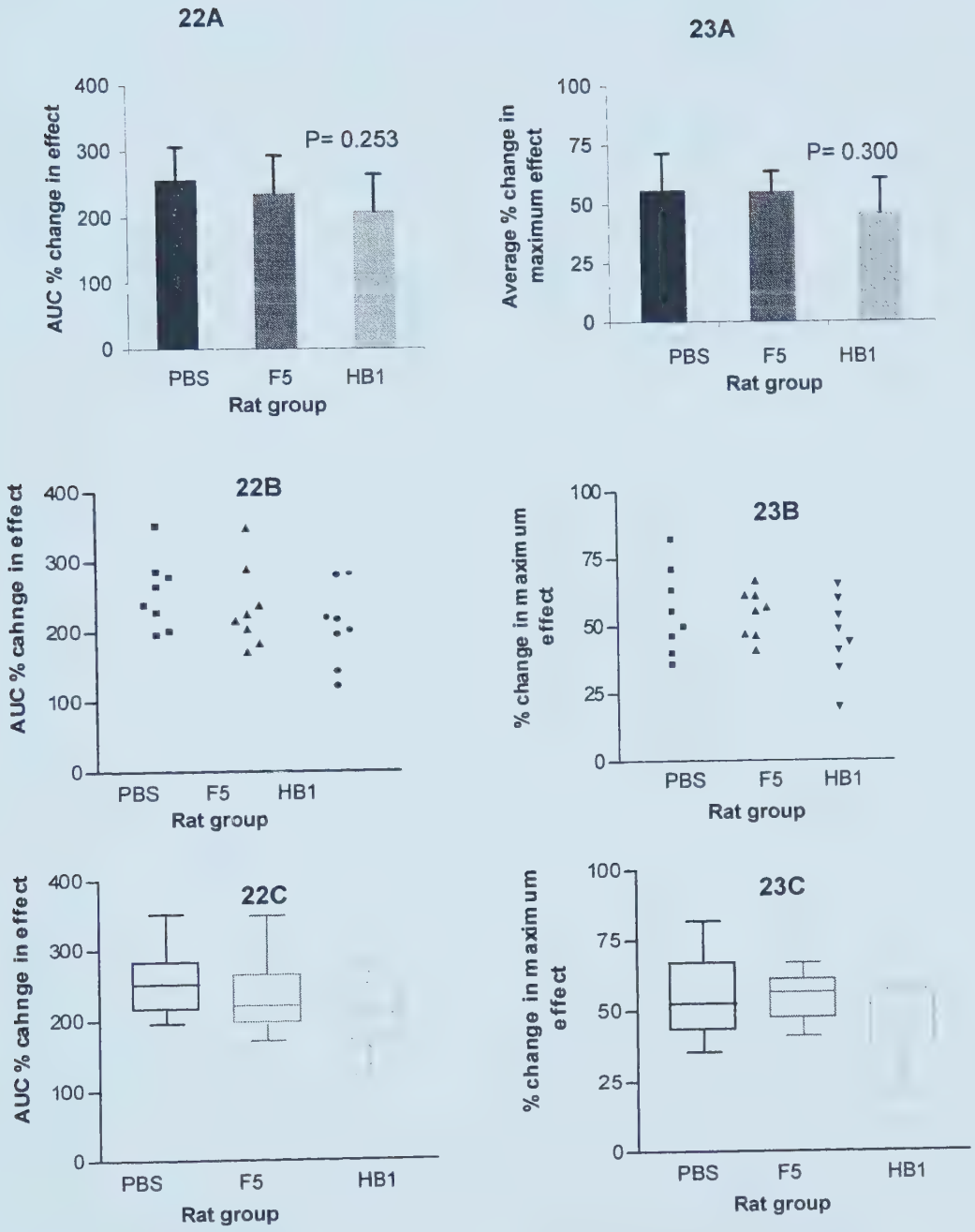
For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method (P= 0.253) or by water displacement method (P= 0.358) was not statistically significant.

#### *3.2.3.2. Immune response*

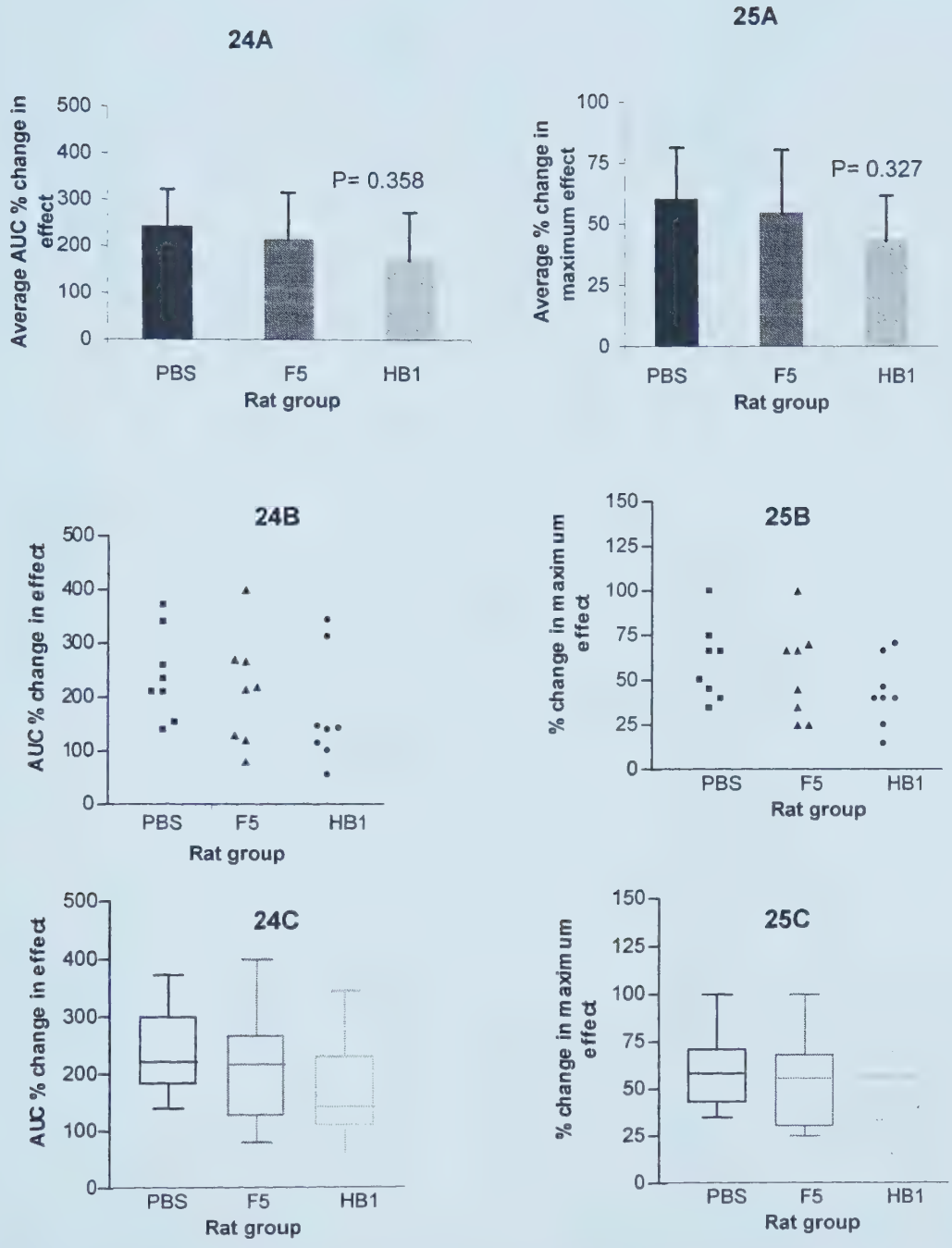
Ab3 was not detected in any of the rat groups tested in the third experiment. RAMA immune response was detected in both the control F5 and the therapeutic HB1 groups as shown in figure 26A.
We correlated the extent of inflammatory response to the level of RAMA immune response in HB1 group as shown in figures 27A-27G. Of particular interest is the demonstration in HB1 rat group of a good correlation between RAMA immune response and the inflammatory response, i.e. the higher the RAMA immune response, the lower the inlammatory response. Surprisingly, some correlation was also observed between the extent of the inflammatory response and the RAMA response in the group treated with F5 mAb as shown in figures 28A- 28G.

### *3.2.4. Experiment 4*

Rats in experiment 4 have been injected four times with 500 μg/rat of either HB1 mAb or have simply received PBS injection via i.v. route (table 5). RAMA and Ab3 responses were measured between each injection at regular intervals as indicated in table 7. Induction and measurement of inflammation was done by the same methods as in

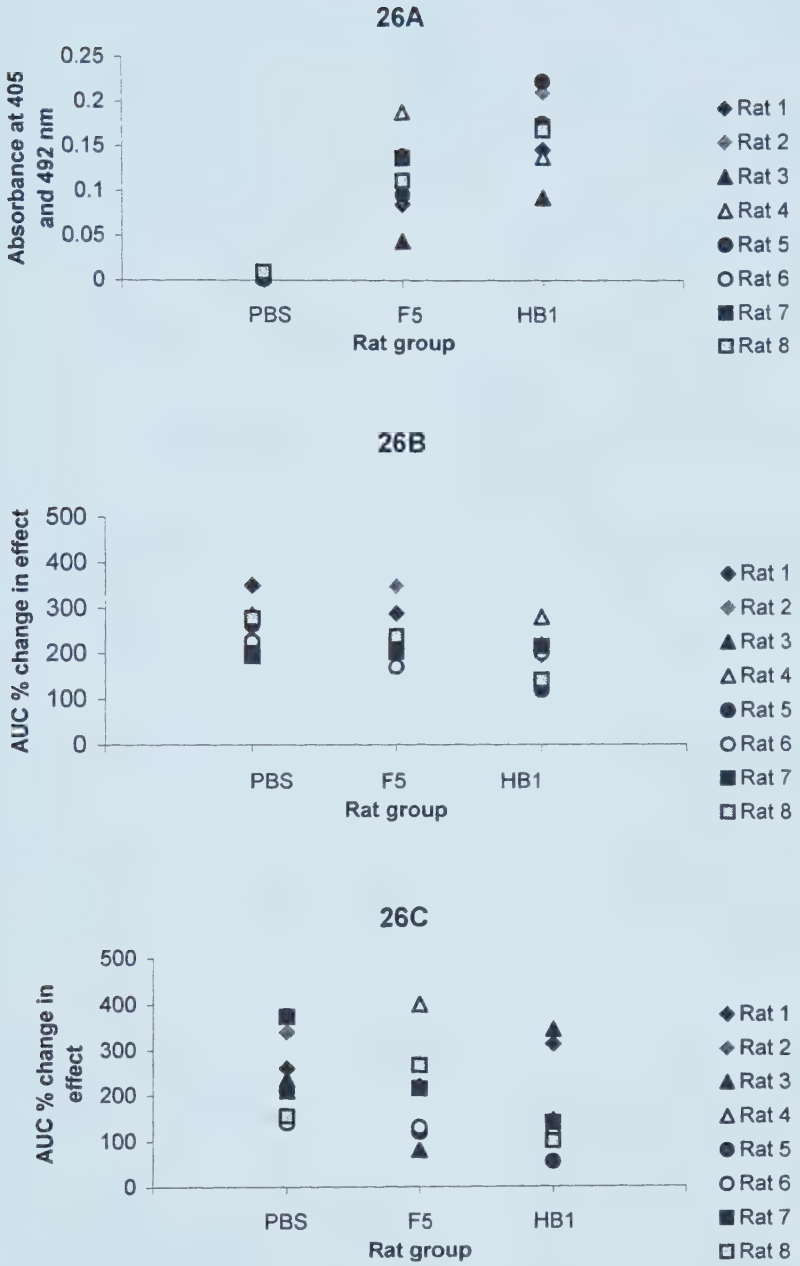



**Figures 22 and 23.** Comparison of the inflammatory response in different rat groups for the third experiment as measured by caliper method.
Rats in experiment 3, that have been immunized with either HB1 mAb, F5 control mAb, or who have simply received PBS injection, sc, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 7 measurements were performed. The results are represented as AUC % change in effect in figures 22A, 22B and 22C; and as % change in maximum effect in figures 23A, 23B and 23C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).




**Figures 24 and 25.** Comparison of the inflammatory response in different rat groups for the third experiment as measured by water displacement method.

Rats in experiment 3, that have been immunized with either HB1 mAb, F5 control mAb, or who have simply received PBS injection, sc, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw volume at regular interval of time, by water displacement method. A total of 7 measurements were performed. The results are represented as AUC % change in effect in figures 24A, 24B and 24C; and as % change in maximum effect in figures 25A, 25B and 25C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).




**Figures 26.** Comparison of the RAMA response with the inflammatory response in different rat groups for the third experiment.

Prior to inflammation induction serum was collected from different rat groups and tested for the presence of RAMA response by the method illustrated in figure 4. The results plotted as column scatter graph in figure 25A represents the extent of the RAMA response in different rat groups. Figures 25B and 25C representing the extent of the inflammatory response measured by caliper and water displacement method respectively have been shown and explained previously as figures 22B and 24B respectively. These two graphs are shown again for the convenience of comparing the extent of the inflammatory response with the level of the immune response in the three rat groups.

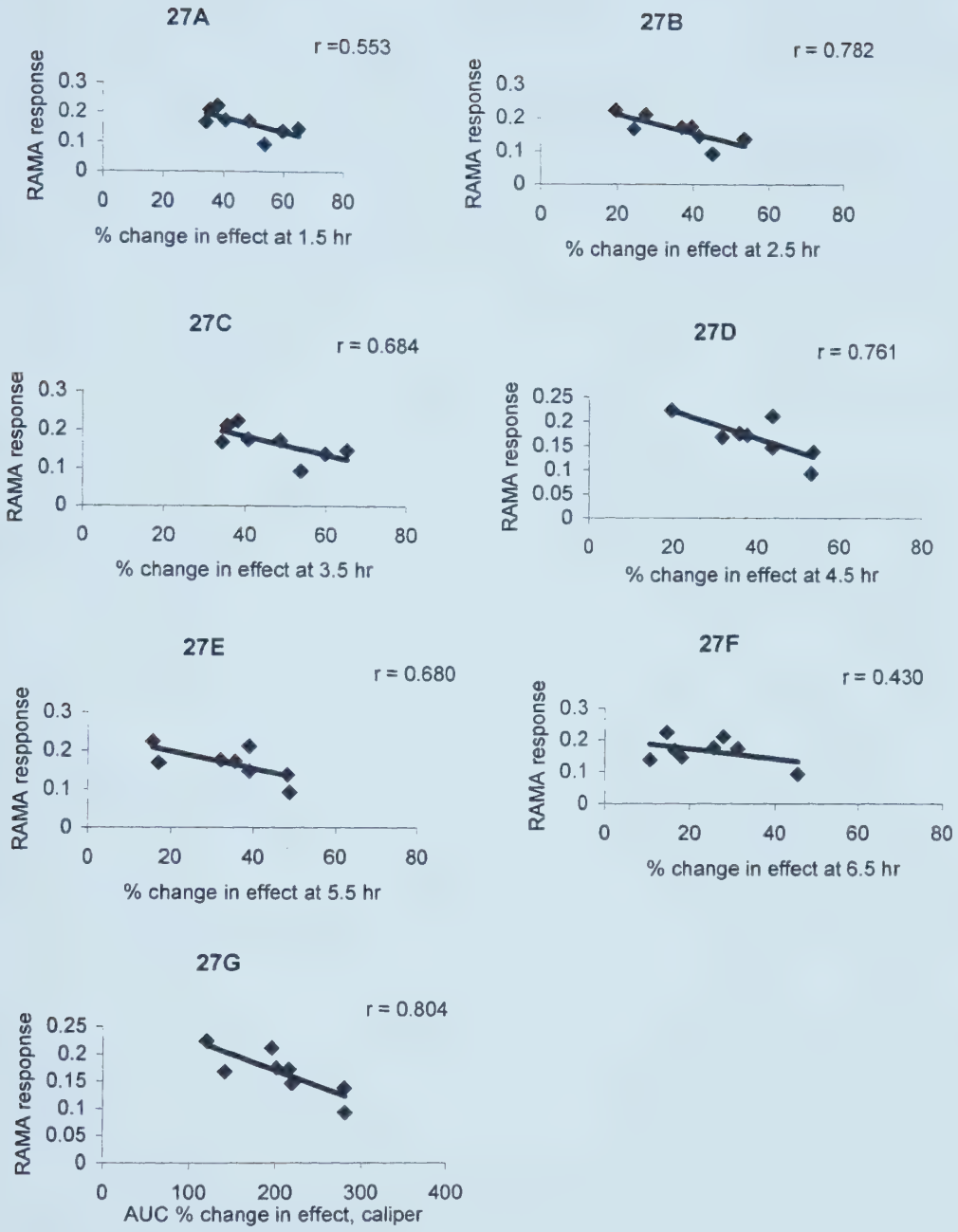



**Figure 27.** Correlation between RAMA response and the inflammatory response at different time measurements in the rat group treated with HB1 antibody in the third experiment.
Rats that have been previously immunized with HB1 mAb were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 7 measurements were performed. Serum was collected from the same group prior to inflammation induction and tested for the peresence of RAMA response. The inflammatory response at different measurement time is plotted as % change in effect against the RAMA response. The inflammatory response was measured at 1.5 hr (A), 2.5 hr (B), 3.5 hr (C), 4.5 hr (D), 5.5 hr (E), 6.5 hr (F). The AUC % change in effect is plotted against RAMA response in graph (G).

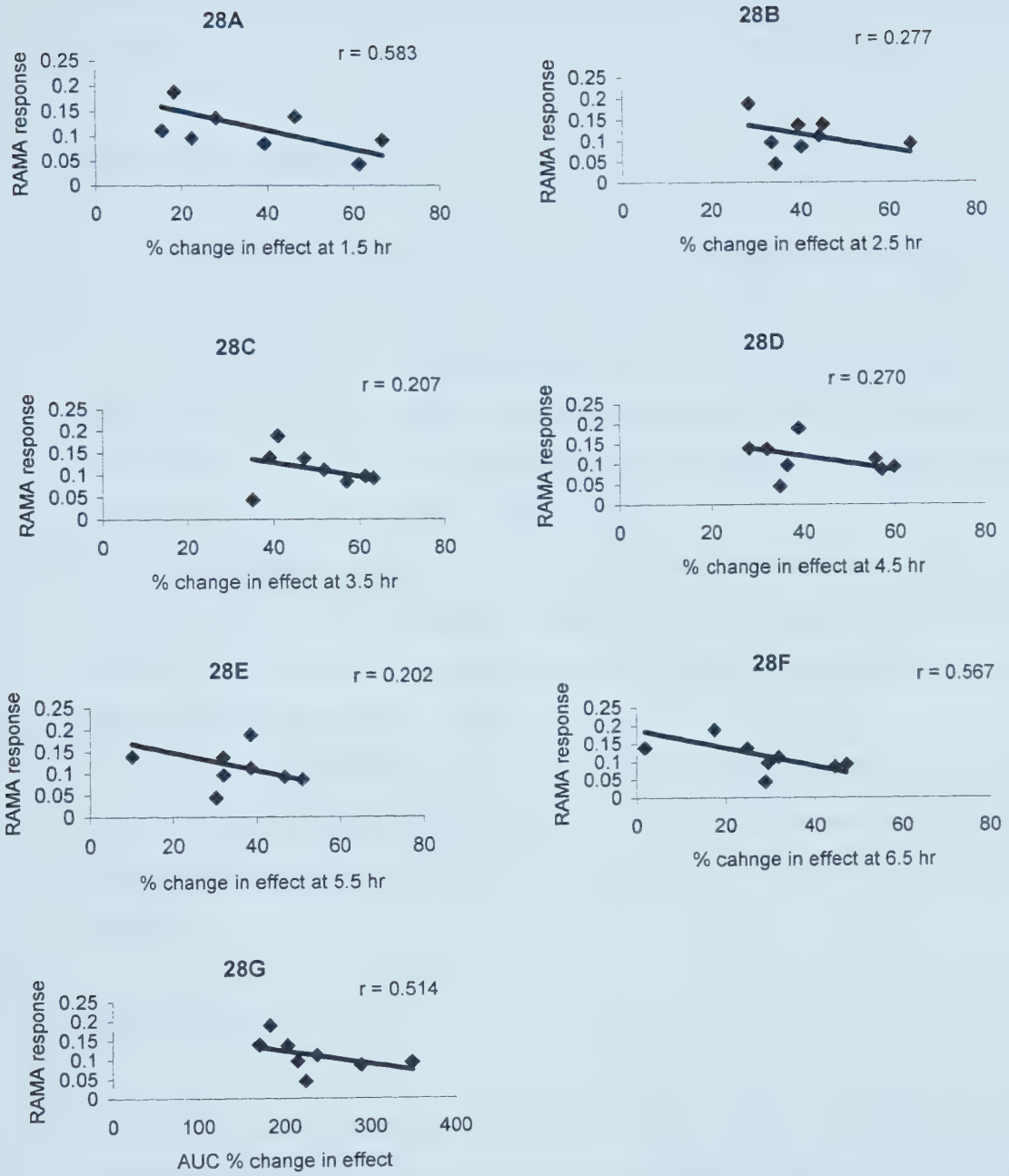



**Figure 28.** Correlation between RAMA response and the inflammatory response at different time measurements in the rat group treated with F5 control antibody in the third experiment.
Rats that have been previously immunized with F5 control mAb were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 7 measurements were performed. Serum was collected from the same group prior to inflammation induction and tested for the peresence of RAMA response. The inflammatory response at different measurement time is plotted as % change in effect against the RAMA response. The inflammatory response was measured at 1.5 hr (A), 2.5 hr (B), 3.5 hr (C), 4.5 hr (D), 5.5 hr (E), 6.5 hr (F). The AUC % change in effect is plotted against RAMA response in graph (G).

experiment 1. Paw edema was observed and measured at intervals of time indicated in table 8.

### *3.2.4.1. Therapeutic effect*

Paw thickness and paw volume measurement results for experiment 4 were analyzed and plotted in the same way as in experiment 1.

Paw thickness measurement results are illustrated as AUC % change in effect in figures 29A, 29B, & 29C; and as % change in maximum effect in figures 30A, 30B, & 30C. Figures 29A, 29B & 29C represent the same data, illustrated in three different ways. The same applies for figures 30A, 30B, & 30C.

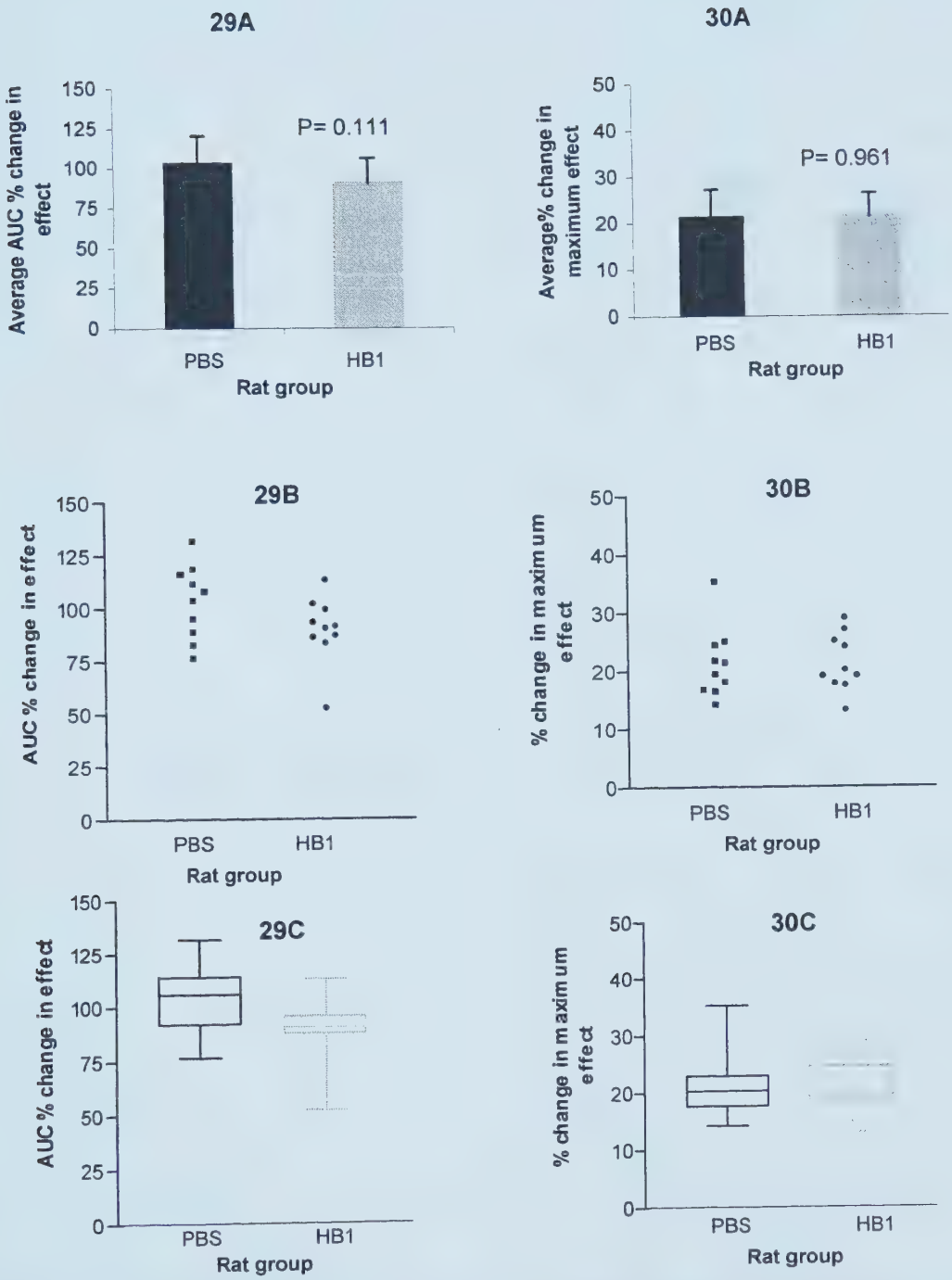
Paw volume measurement results are illustrated as AUC % change in effect in figures 31A, 31B, & 31C; and as % change in maximum effect in figures 32A, 32B, & 32C. Figures 31A, 31B, & 31C represent one graph, illustrated in three different ways. The same applies for figures 32A, 32B, & 32C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method ($P= 0.111$) or by water displacement method ($P= 0.188$) was not statistically significant.

### *3.2.4.2. Immune response*

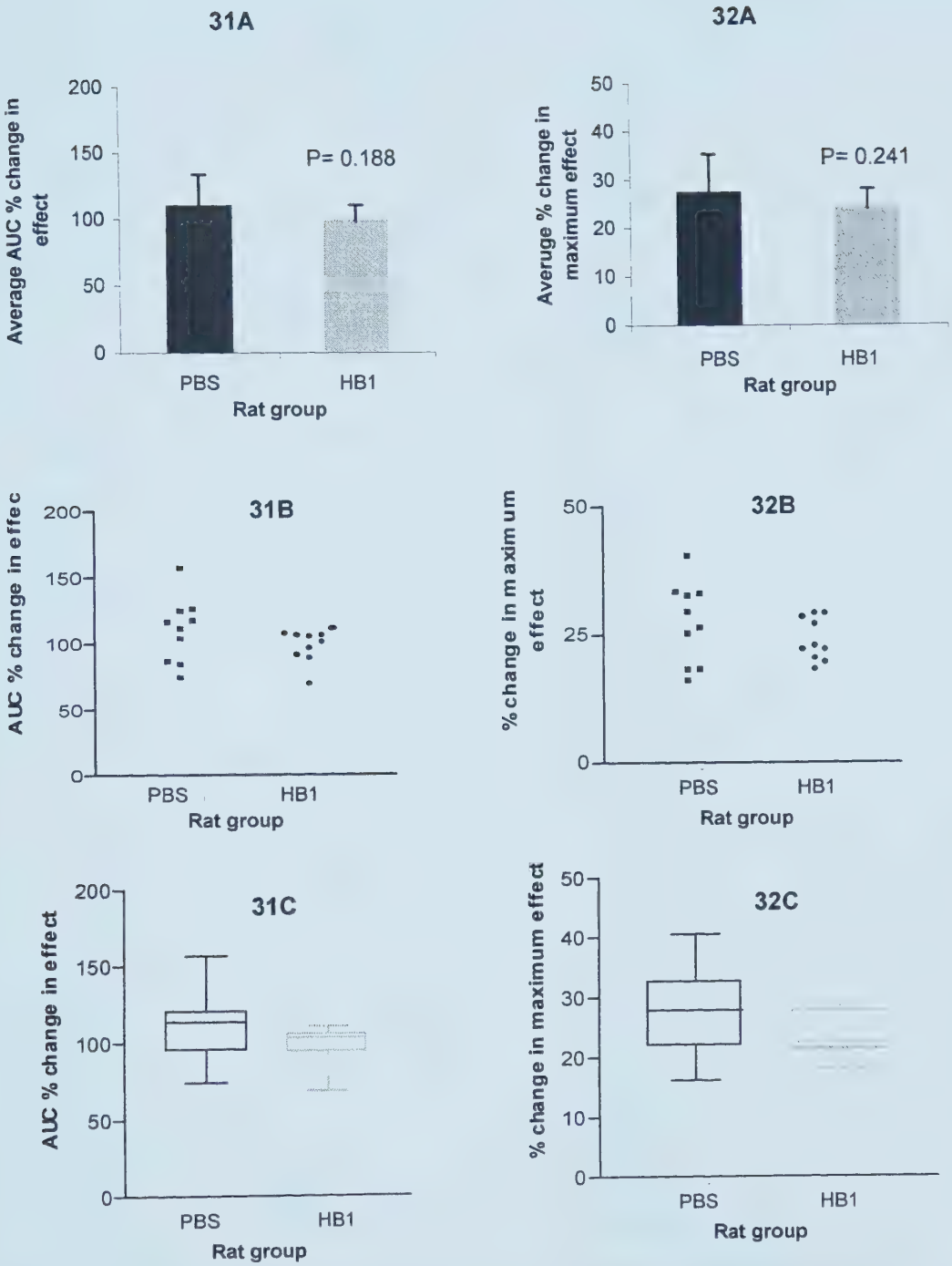
Ab3 was not detected in any of the rat groups tested in the fourth experiment. The RAMA immune response was detected in one rat that belongs to the therapeutic HB1 group as shown in figure 33A.

AUC % change in effect from the measurements of caliper and water displacement methods showed that one rat in the therapeutic HB1 group had responded with minimal inflammatory response as shown in figures 27B and 29B respectively. Therefore, we compared the extent of the inflammatory response figures 33B and 33C to the level of RAMA immune response in each rat in figure 33A. In fact, the same rat that has shown the highest RAMA immune response as shown in figure 33A has also shown




**Figures 29 and 30.** Comparison of the inflammatory response in different rat groups for the fourth experiment as measured by caliper method.

Rats in experiment 4, that have been previously immunized with either HB1 mAb or who have simply received PBS injection, iv, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 29A, 29B and 29C; and as % change in maximum effect in figures 30A, 30B and 30C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).




**Figures 31 & 32.** Comparison of the inflammatory response in different rat groups for the fourth experiment as measured by water displacement method.

Rats in experiment 4, that have been previously immunized with either HB1 mAb or who have simply received PBS injection, iv, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw volume at regular interval of time, by water displacement method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 31A, 31B and 31C; and as % change in maximum effect in figures 32A, 32B and 32C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).

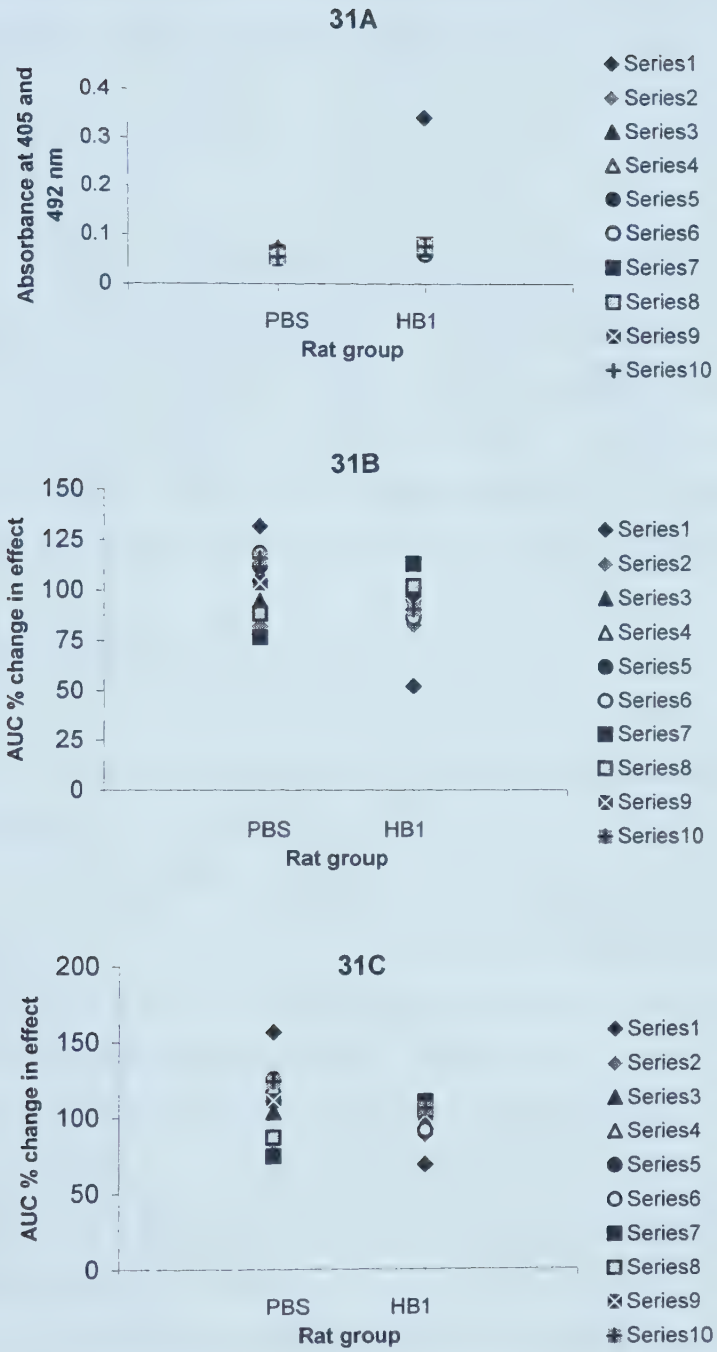



**Figure 33.** Comparison of the RAMA response with the inflammatory response in different rat groups for the fourth experiment.

Prior to inflammation induction serum was collected and tested for the presence of RAMA response by the method illustrated in figure 4. The results plotted as column scatter graph in graph 31A represents the extent of RAMA response in different rat groups. Figures 33B and 33C representing the extent of the inflammatory response neasured by caliper and water displacement method respectively have been shown and explained previously as figures 29B and 31B respectively. These two graphs are shown again for the convenience of comparing the extent of the inflammatory response with the level of the immune response in the three rat groups.

the least inflammatory response measured by both caliper and water displacement methods as shown in figures 33B and 33C respectively.

### *3.2.5. Experiment 5*

Rats in experiment 5, have been injected four times with 500 μg/rat of either KLH-conjugated HB2, control KLH-conjugated Flopc-21 mAbs, or have simply received PBS injection via ip route (table 5). RAMA and Ab3 immune responses were measured between each injection as indicated in table 7. Induction and measurement of inflammation were done by the same methods as in experiment 1. Paw edema was observed and measured at intervals of time indicated in table 8.

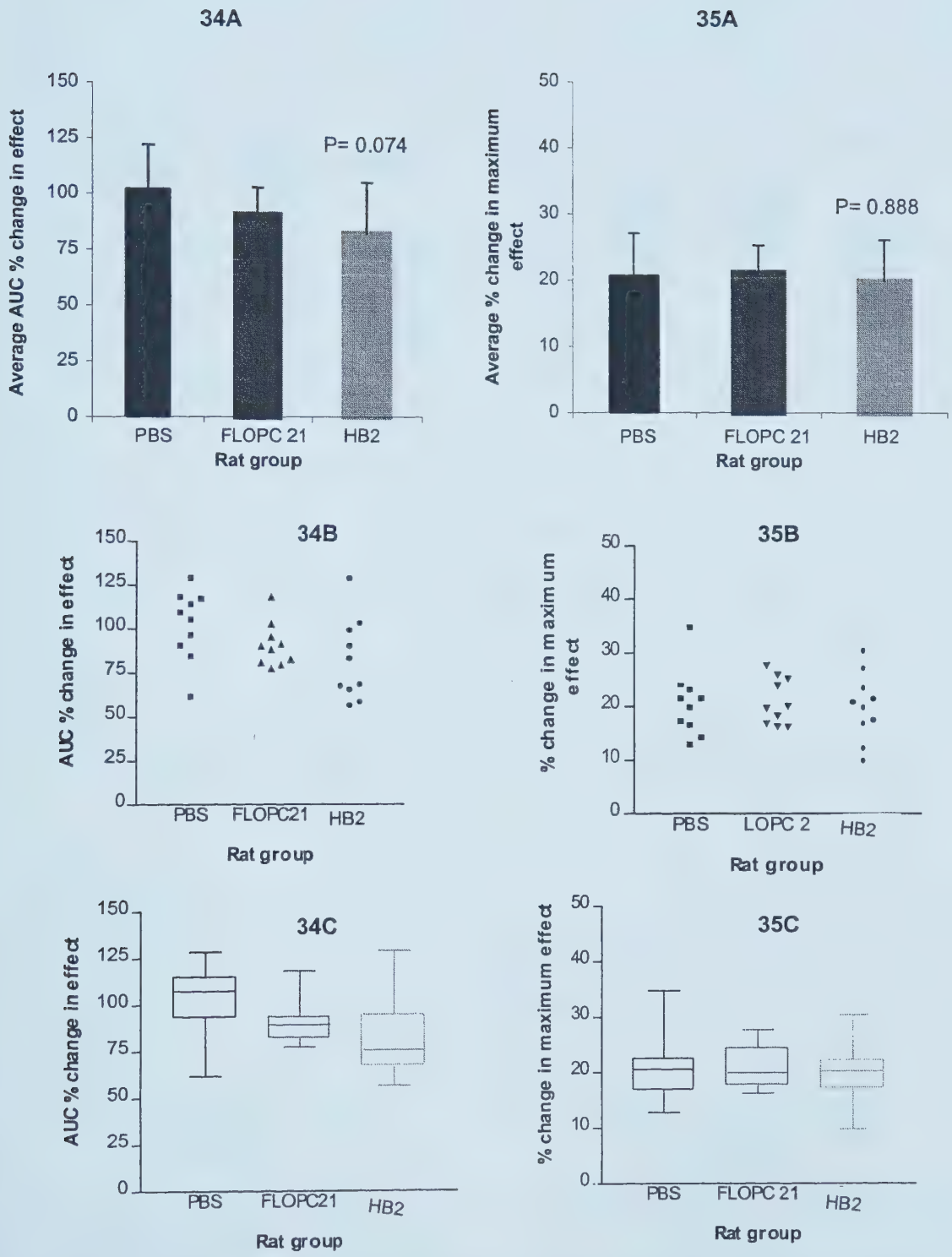
#### *3.2.5.1. Therapeutic effect*

Paw thickness and paw volume measurement results were analyzed and plotted in the same way as in experiment 5.

Paw thickness measurement results are illustrated as AUC % change in effect in figures 34A, 34B, & 34C; and as % change in maximum effect in figures 35A, 35B, & 35C. Figures 34A, 34B & 34C represent the same data, illustrated in three different ways. The same applies for graphs 35A, 35B, & 35C.

Paw volume measurement results are illustrated as AUC % change in effect in figures 36A, 36B, & 36C; and as % change in maximum effect in figures 37A, 37B, & 37C. Figures 36A, 36B, & 36C represent one graph, illustrated in three different ways. The same applies for figures 37A, 37B, & 37C.

For both paw thickness and paw volume measurements the least edema was observed in HB1 group, but this difference in change in effect measured by caliper method ($P=0.074$) or by water displacement method ($P= 0.065$) was not statistically significant.




**Fgures 34 and 35.** Comparison of the inflammatory response in different rat groups for the fifth experiment as measured by caliper method.

Rats in experiment 5, that have been previously immunized with either HB2 mAb, Flopc-21 control mAb, or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw thickness at regular interval of time, by caliper method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 34A, 34B and 34C; and as % change in maximum effect in figures 35A, 35B and 35C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).

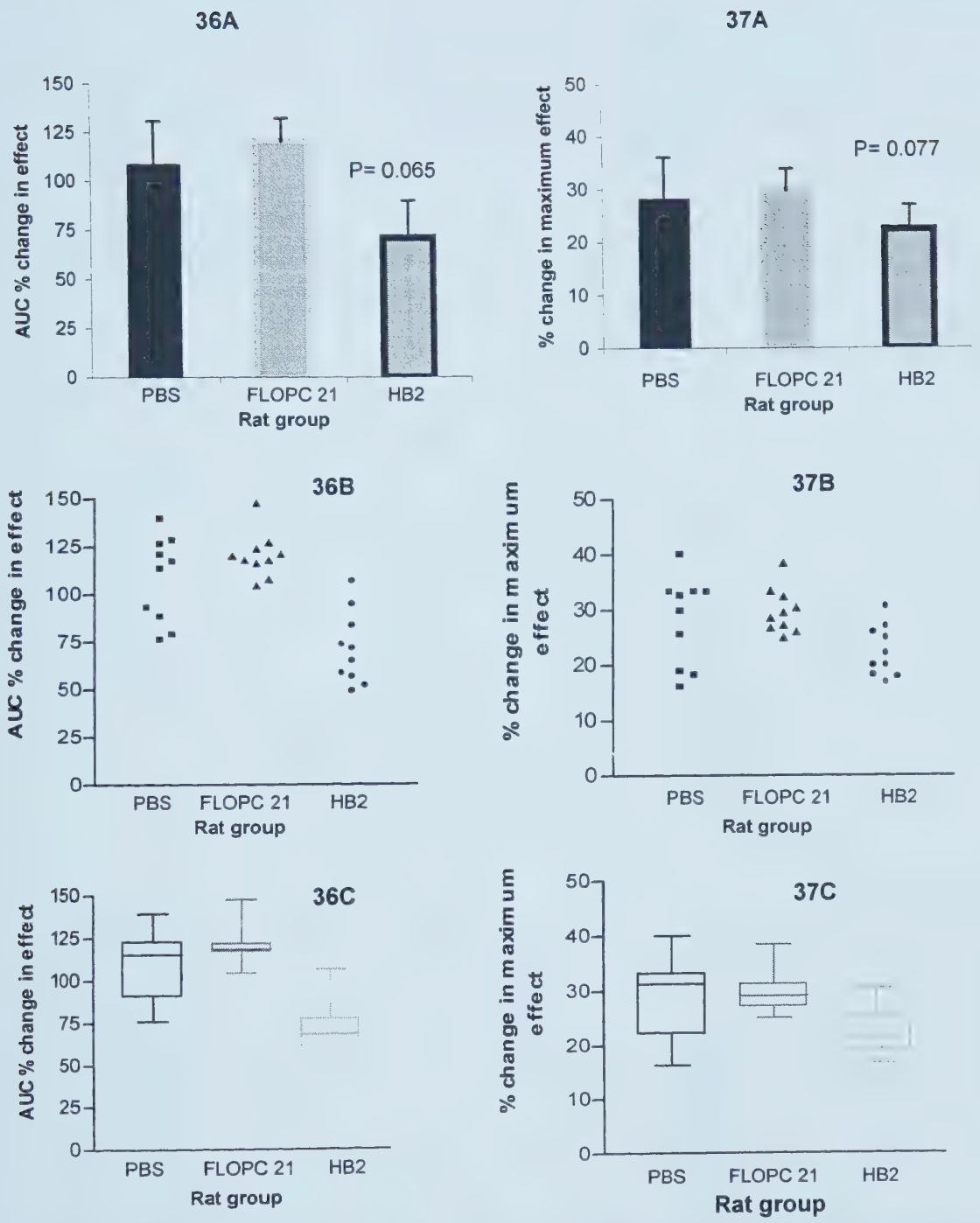
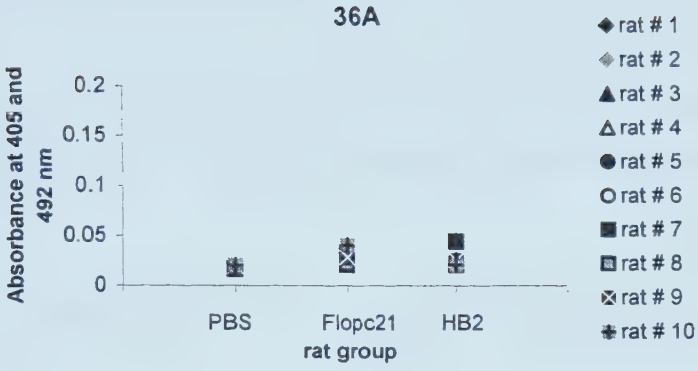



**Figure 36 and 37.** Comparison of the inflammatory response in different rat groups for the fifth experiment as measured by water displacement method.

Rats in experiment 5, that have been previously immunized with either HB2 mAb, Flopc-21 control mAb, or who have simply received PBS injection, ip, were subjected to acute inflammation in the right hind paw by carrageenan injection. The extent of the inflammatory response was determined by measuring paw volume at regular interval of time, by water displacement method. A total of 8 measurements were performed. The results are represented as AUC % change in effect in figures 36A, 36B and 64C; and as % change in maximum effect in figures 37A, 37B and 37C. The results are plotted as bar graphs (A), column scatter (B) and whisker box (C).





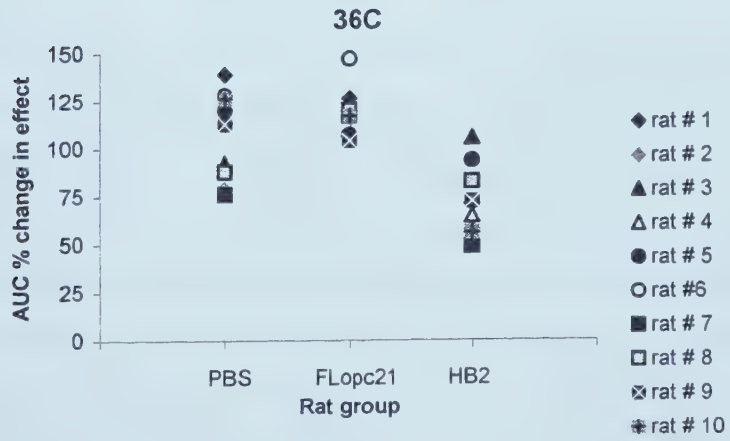



**Figure 38.** Comparison of the Ab2 response with the inflammatory response in different rat groups for the fifth experiment.

Prior to inflammation induction serum was collected and tested for the presence of RAMA response by the method illustrated in figure 4. The results plotted as column scatter graph in figure 36A represents the extent of the RAMA response in different rat groups. Figures 38B and 38C have been shown and explained previously as figures 34B and 36B respectively. These two graphs are shown again for the convenience of comparing the extent of the inflammatory response with the level of the immune response in the three rat groups.

#### *3.2.5.2. Immune response*

Ab3 was not detected in any of the rat groups tested in the fifth experiment. All tested groups have shown minimal (background) RAMA immune response as shown in figure 38A.

## *3.3. Elucidation of the mechanism of action of therapeutic mAbs*

### *3.3.1. Binding of monoclonal antibodies to Leukocytes*

The binding of HB1, HB2 and control mAbs to PMNL and lymphocyte was studied by FACScan and fluorescent microscopy. The binding of HB1, HB2 and control mAbs to HUV-EC-C cells was studied by fluorescent microscopy. Examples of histograms obtained by FACS analysis are shown in figure 39. All tested mAbs did not show significant binding to human lymphocytes, neither to rat neutrophils nor to HUVEC cells.

### *3.3.2. In vitro inhibition of leukocyte rolling on microchips*

This study was realized in collaboration with Dr Richard Smith at the Alberta Research Council (Edmonton, Alberta, Canada). For this study we used a microchip glass wafer device that consists of channels in which we can observe in vitro cell rolling and adhesion under flow conditions, the process that mimics the in vivo cell rolling and adhesion events. The microchip device was coated with either recombinant human E- or P- selectin as described in section 2.11, and used to study the effect of different agents on the rolling of human white blood cells. We intended to carry this assay with both rat and human leukocytes on recombinant human E- and P-selectin coated channels, However, there was no consistent rolling of rat leukocytes on the recombinant human E- or P-selectin coated channels. For this reason and for the reason that rat E- and P-selectins were not available, the assay was carried only with human leukocytes. The images of cell rolling and inhibition steps were captured at different times and the rolling cells were counted on both sides of the channel (figure 40). The effect of each of the tested agents

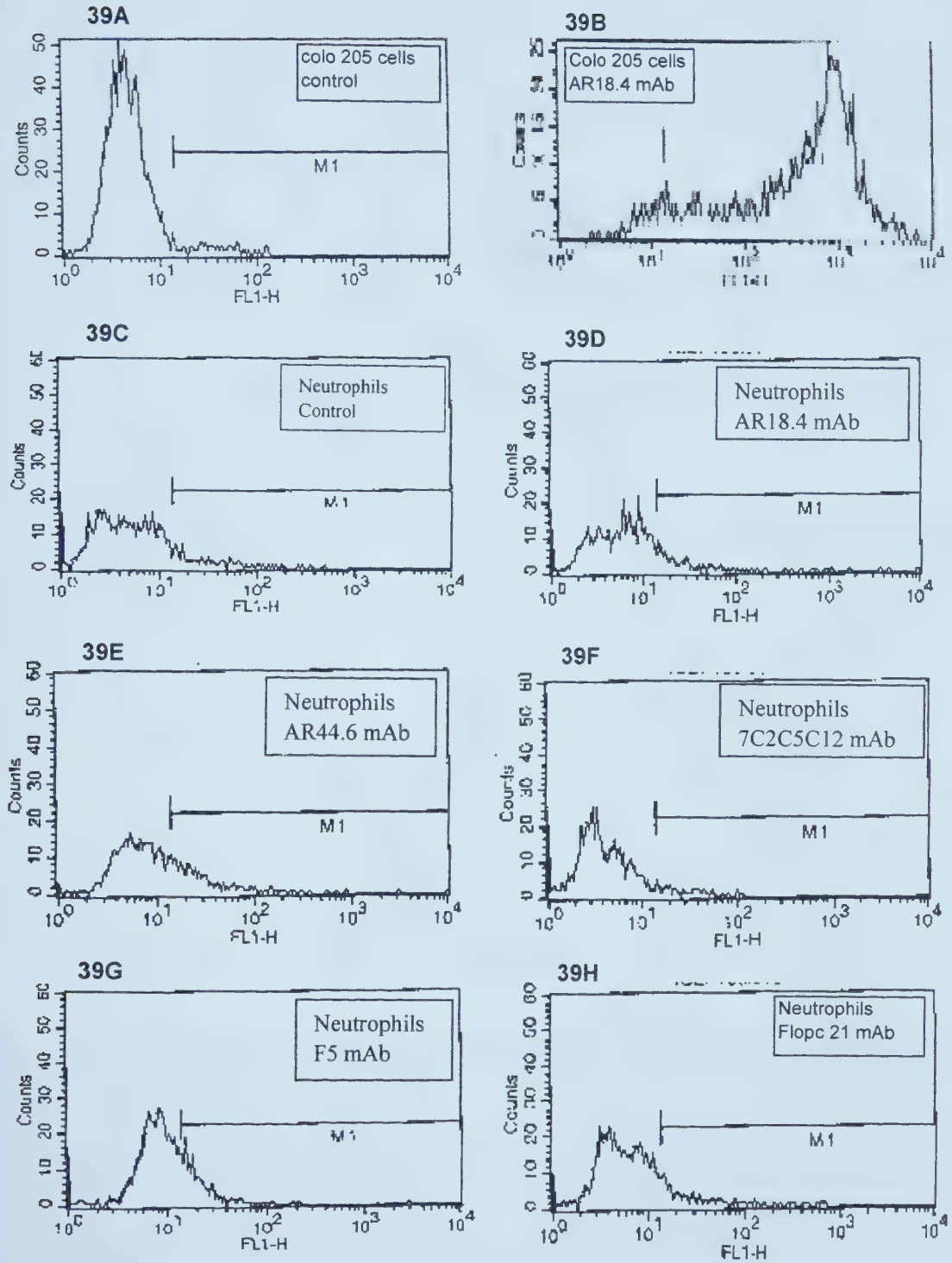



**Figure 39.** Binding of therapeutic AR18.4 (IgM) and AR44.6 (IgG3) and control F5 (IgM), 7C2C5C12 (IgM) and Flopc-21 (IgG3) monoclonal antibodies to human neutrophils as determined by single step indirect immunofluorescence flow cytometer . For figures 39A and 39B colo 205 cells were used as positive control. Sialyl Lewis A epitope is known to be expressed at the surface of these cells.

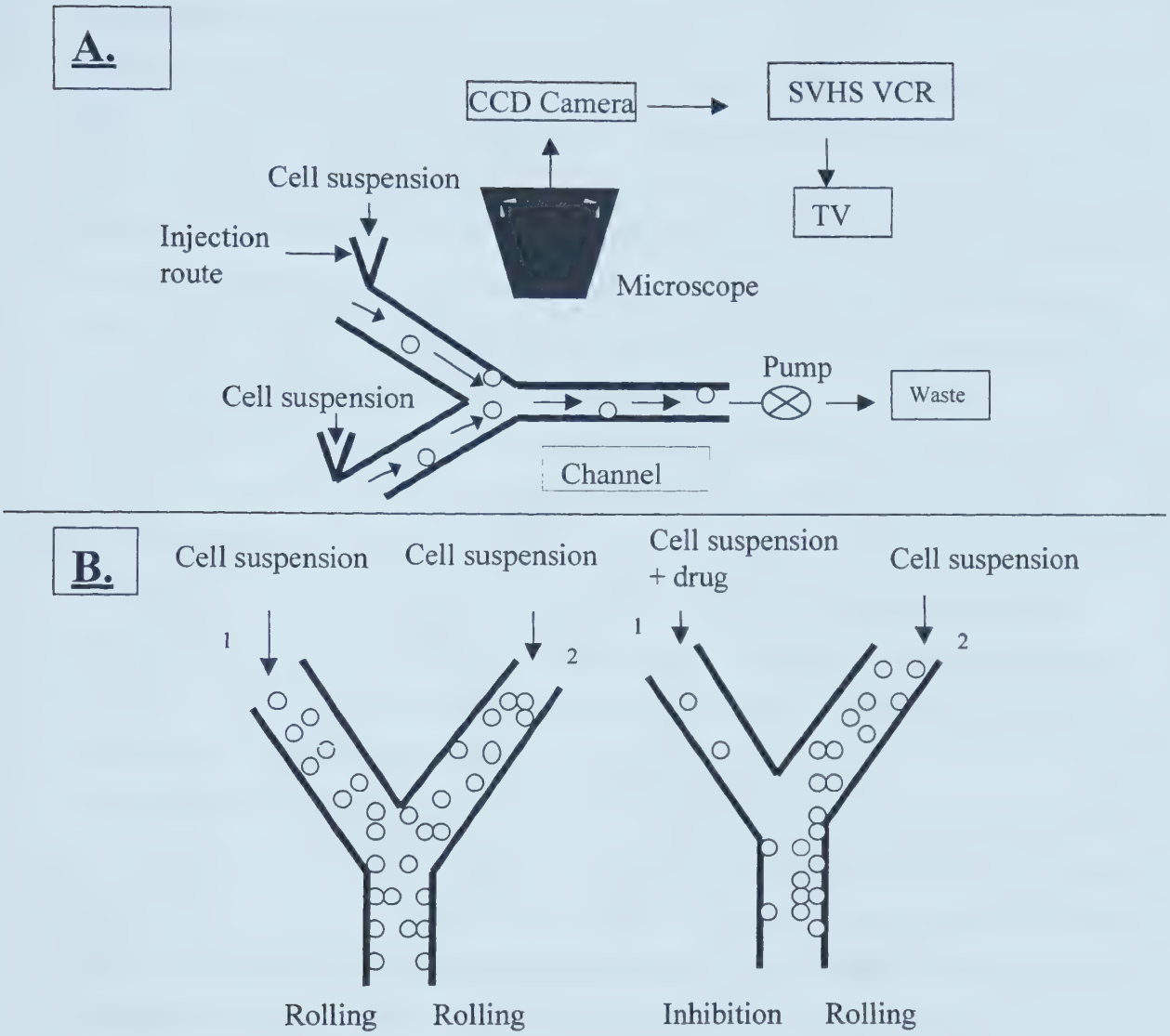



**Figure 40**. A: Illustration of the instrumental setup of E- and P-selectin coated channels used for thein vitro inhibition of leukocyte rolling in microchips. B. Schematic diagram of cell adhesion and inhibition steps in E- or P-selectin coated chips.
PMNLsuspension was added to side 1 and side 2 of the channel through the injection route, and allowed to run at a flow rate of 2.5 ml/min and under a negative pressue regulated by a microsyringe pump. The cell suspension flow was regulated by a luminal fluidic flow that prevented the cells to diffuse from one side of the channel to the other. When the rolling step was achieved, the drug under study was added to side 1 of the channel, and side 2 was used as a control channel. The pump sucked the added material into the channel to start the inhibition step. The images of cell rolling and inhibition steps were captured at different times, and the number of cells was counted in both sides of the channel

was determined as the percentage of rolling human PMNL from those in the control channel (100%) in which no agents were added.

Normal human serum did not cause a reduction in human PMNL rolling on E- or P-selectin coated channels as shown in figures 41 and 42 respectively. The same experiment performed with normal rat serum instead of normal human serum revealed by microscopic observation that such treatment resulted in an increased adherence of a small number of leukocytes on both E- and P- selectin coated channels. This effect however was very mild and did not affect the ro[illegible] 82 [illegible]f cells on either E- or P- selectin coated channels as shown in figures 41 and 42 respectively.

Anti-E- and anti P-selectin mAbs were used to demonstrate effective rolling inhibition of PMNL on E- and P- selectin coated channels respectively. There was only 1,4 % of rolling cells observed with the addition of anti-E- selectin mAb, as compared to the side with the control rolling cells as shown in figure 41, and 14,4% rolling cells with the addition of anti-P-selectin mAb as shown in figure 42. Therefore, the rolling of human PMNL leukocytes on recombinant human E- and P- selectin coated channel was inhibited significantly by Anti-E- and anti-P- selectin mAbs (Positive control).

It was of particular importance to observe that the addition of 1:10 dilution of the RAMA positive serum obtained from rats immunized with HB1 antibody (rat # 2, 4, 5, 6, 7 and 8 collectively, experiment # 3) has caused a significant inhibitory effect on PMNL rolling on both E-selectin and to P- selectin coated channels. In fact, the HB1 rat serum was able to reduce the rolling of PMNL on E-selectin coated channels to 0% resulting in a 100 % inhibition ($P< 0.001$). The same rat serum resulted in a 76 % inhibitory effect on P-selectin coated channels ($P< 0.001$), since it was able to reduce the rolling of PMNL on P-selectin coated channels from 100 % to 23.2 %. In opposition to normal serum no change in cell adherence or cell morphology was observed with the addition of rat serum immunized with HB1 mAb.

Unexpectedly, the RAMA positive control F5 rat serum also showed some inhibition of the rolling of PMNL leukocytes on recombinant human E- and P-selectin coated channels. However, this inhibition was considerably less marked than the one observed with the therapeutic mAb. The addition of serum obtained from rats immunized with F5 antibody who were positive for RAMA response (rats # 4, 6 and 7 collectively,

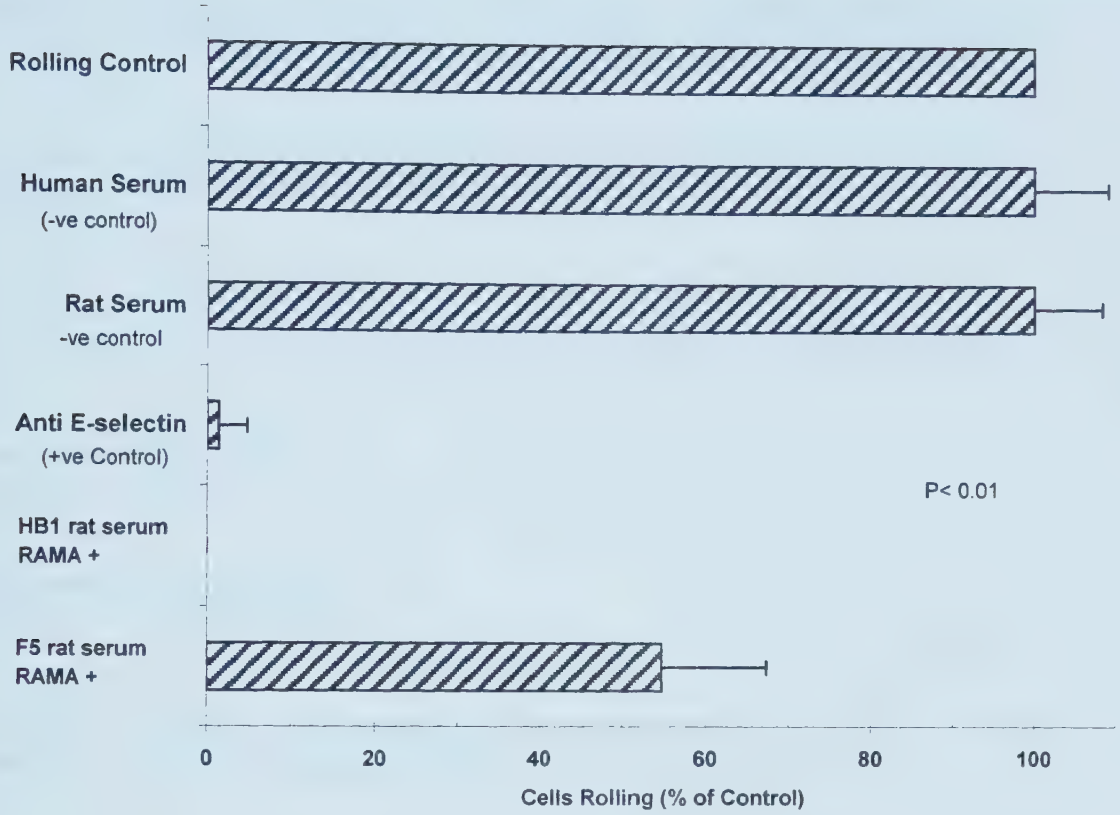



**Figure 41.** The effect of different agents on human leukocyte rolling in microchip channels coated with recombinant human E-selectin.

The microchip channels were coated with 20 μg/ml of E- selectin, then 1x $10^7$ cells/ml of human neutrophils in RPMI, 5% FCS were pumped through the channel at a flow rate of 2.5 μl/min so the rolling step is acheived. The following agents were tested at a volume of 10 μl and at a dilution of 1:10 v/v in RPMI: normal fresh human serum (negative control), normal fresh rat serum (negative control), HB1 rat serum and F5 rat serum . 10 μl of 100 mg/ml of anti-E-selectin antibody was used as a positive control. The above mentioned samples were added to different channels. HB1 rat serum was collected from rats # (2, 4,5, 6,7 and 8 collectively) who showed RAMA response after immunization with HB1 mAb in the third animal experiment. F5 rat serum was collected from rats # (4, 6 and 7 collectively) who showed RAMA response after immunization with F5 control mAb in the third animal experiment.The graph bars show percentage of human PMNL rolling on E-selectin coated microchip channels.

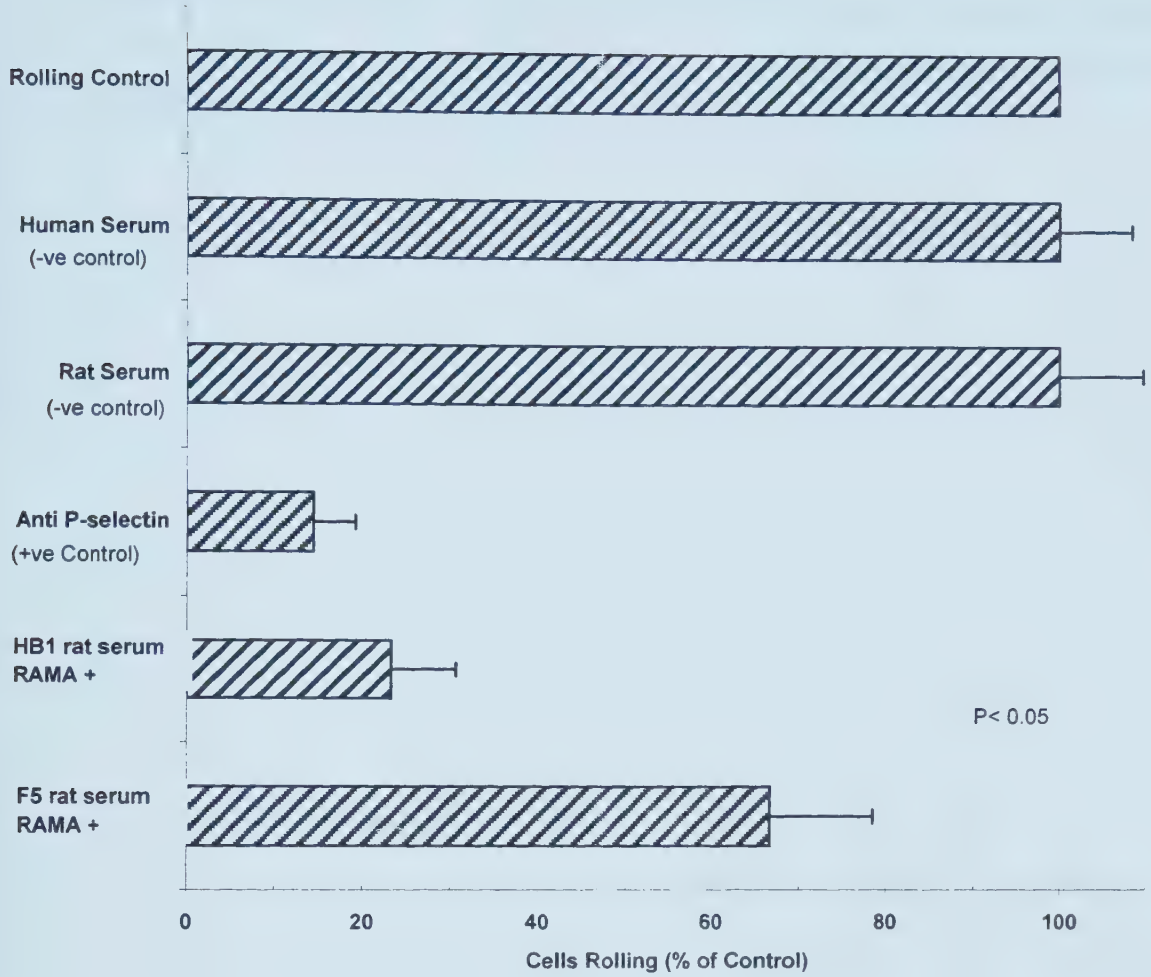



**Figure 42.** The effect of different agents on human leukocyte rolling in microchip channels coated with recombinant human P-selectin.

The microchip channels were coated with 20 μg/ml of P- selectin, then 1x $10^7$ cells/ml of human neutrophils in RPMI, 5% FCS were pumped through the channel at a flow rate of 2.5 μl/min so the rolling step is acheived. The follwing agents were tested at a volume of 10 μl and at a dilution of 1:10 v/v in RPMI: normal fresh human serum (negative control), normal fresh rat serum (negative control), HB1 rat serum and F5 rat serum . 10 μl of 100 mg/ml of anti-P-selectin was used as a positive control. The above mentioned samples were added to different channels. HB1 rat serum was collected from rats # (2, 4,5, 6,7 and 8 collectively) who showed RAMA response after immunization with HB1 mAb in the third animal experiment. F5 rat serum was collected from rats # (4, 6 and 7 collectively) who showed RAMA response after immunization with F5 control mAb in the third animal experiment.The graph bars show percentage of human PMNL rolling on P-selectin coated microchip channels.

experiment # 3) caused a 45 % reduction in PMNL rolling on E-selectin coated channels ($P < 0.001$); and a 33 % reduction in PMNL rolling on P-selectin coated channels ($P < 0.001$) as shown in figures 41 and 42 respectively.

# CHAPTER 4. DISCUSSION

## *4.1. Disccussion of animal experiments*

The tumor antigen CA19-9 against which the therapeutic mAbs (HB1 and HB2) were raised is a high molecular weight mucin-type glycoprotein. The sialylated lactofucopentaose $SLe^a$ carbohydrate is the main epitope for CA19-9 antigen (Hamanka et al., 1996; Rye et al., 1998). Such epitope is a well known tumor marker associated with poor prognosis in gastrointestinal malignancies such as pancreatic, gall bladder, gastric and colorectal cancers (Rye et al., 1998; Ravindranath et al., 1997). It has also been documented that the $SLe^a$ epitope is normally expressed in bile and saliva (Zopf and Hansson, 1988).

In four animal experiments, the therapeutic mAb (HB1) was tested. Variability in those experiments include route of immunization (ip, s.c, i.v.), dose of injected mAb (250 or 500 μg/rat), form of mAb tested (conjugated and unconjugated) and the use of an adjuvant (QUIL A). In one experiment the therapeutic mAb HB2 was tested.
In order to evaluate the ability of both HB1 and HB2 mAbs in inhibiting inflammatory process we chose carrageenan-induced inflammation.

An immune RAMA response against the injected Abs was detected at variable levels in all experiments. RAMA can be directed against the isotypic, allotypic and idiotypic determinants of the injected mAbs. Competitive assays were carried out for the determination of a specific anti-idiotypic response in rats that showed positive RAMA response. Results (not shown) from the different competitive assays were either not consistant or not specific for the idiotypic response. Those assays were not pursued further due to the high cost or difficulty in purification of the competitive agents (CA19-9 or $Sle^a$).

Ab3 could not be detected in any of the experiments. The inability to detect Ab3 in rat serum could be simply due to the fact that there is no Ab3, but it should be also considered that Ab3 may be bound to Ab2. The high affinity of Ab3 for Ab2 and the low Ab3 response will lead to undetectable Ab3.

In the first experiment only one rat (rat # 5) had a high RAMA response as shown in figure 15A. Of particular interest, this specific rat also had the lowest inflammatory response as shown in figures 15B & 15C, suggesting that the low inflammatory response is due to the effect of the high RAMA response. On the other hand, rat (# 2) has shown the highest inflammatory response as illustrated in figures 15B & 15C and it also has had the poorest RAMA immune response as presented in figure 15A.

For the purpose of inducing a stronger immune response, we increased the dose of the tested mAbs in the second experiment. In this experiment we see a high immune response in the HB1 group compared to the other tested groups, however, no significant correlation between the therapeutic efficacy and the RAMA immune response could be found (figure 21). The absence of correlation between the inflammatory and the humoral immune responses may be due to the lack of specificity of the method used for Ab2 detection since the assay used actually measures RAMA response. It is worth to mention that the tested antibodies in both experiments 1 and 2 were conjugated with KLH. KLH conjugation increases the immunogenecity of the mAbs. However, such treatment may also affect the specificity of the Abs produced by the host in response to the immunization with the KLH-conjugated mAb. Indeed, subsequent to the covalent attachment of KLH to mAb, number of antigenic determinants may be masked including idiotypic, isotypic and allotypic determinants. In the first two experiments the antibody's binding site was partially masked by KLH which resulted in a great loss of the Abs binding activity as shown in table # 5.

For the purpose of retaining the binding activity of the tested mAbs and enhancing the anti-idiotypic immune response, unconjugated Abs were injected into rat groups subcutaneously in the third experiment. QUIL A adjuvant was used to enhance the immune response. Subsequently, a high RAMA response was observed, with the highest RAMA response observed once more in the HB1 group as shown in figure 26A. Of particular interest, we observed a good correlation between the extent of the inflammatory response and the RAMA immune response level in the group treated with HB1 mAb. This correlation was observed at almost all points of time when inflammation measurement was taken as shown in figures 27A-27G which suggest again that the inflammatory response is dependent on the level of RAMA response. We believe that rat

immunization with unconjugated form of mAb has resulted in enhancing the anti-idiotypic immune response and reducing the inflammatory response to a great extent. Surprisingly, a correlation between RAMA response and the therapeutic effect was also observed in the control mAb group. Although this effect was less significant than in the therapeutic group, it suggests that the induction of RAMA response may be beneficial.

In the fourth experiment we injected rats with unconjugated therapeutic HB1 mAb, intravenously, so for future clinical studies, it is likely that i.v injection will be preferred compared to s.c injection. In the rat group treated with anti-$SLe^a$ mAb one rat (rat #1) has shown the highest immune RAMA response as shown in figure 33A. Of particular interest, this one rat is the same rat that had the least inflammatory response measured by both caliper and water displacement methods as shown in figures 33B and 33C respectively. The intravenous injection of the therapeutic mAb has not resulted in a much better effect than the intraperitoneal route for reasons that we believe are related to the absence of adjuvant and to laboratory technical difficulties that accompanied the intravenous injection procedure.

In addition to the four experiments mentioned above, we carried out a fifth experiment where rat groups were injected ip either with a therapeutic KLH-conjugated HB2 mAb, control KLH-conjugated FLOPC-21 mAb, or PBS. HB2 mAb has the same binding specificity as HB1 Ab but is an $IgG_3$ Ab. It is likely that the epitope recognized by Flopc-21 is different than the one recognized by HB1 or HB2.

In this experiment, the group that was treated with HB2 mAb responded to a very little extent with less inflammatory response than the control groups, as shown by caliper and water displacement method results illustrated in figures 34-35 and 36-37 respectively. We were not able to correlate between the therapeutic efficacy of HB2 mAb and the rats' immune response level since RAMA response was not detected in any of the tested groups as shown in figure 38A. There was a great loss of the antibodies binding activity as shown in table # 5. We believe that the binding site of the Ab was masked by KLH, therefore this resulted in impaired Ab2 response.

We have shown that the obtained therapeutic effect is variably correlated with the RAMA immune response that was detected in rat serum and we assume that the humoral immune response is responsible for the obtained therapeutic effect in the studied

inflammatory model. Unlike anticancer treatment in which both humoral and cellular responses were induced, we assume that MHC cell response can not be induced with $SLe^a$ specific mAb treatment for few reasons. First, unlike peptides that can be processed and presented to MHC molecule, $SLe^a$ is a carbohydrate molecule and not a peptide, and therefore, as a sugar, it can not induce an MHC restricted T-cell immune response. Second, as mentioned in section 1.1.2, we believed that the induction of a cellular immune response is highly dependent on the interaction of the injected antibody with the circulating antigen. Unlike Sialyl $Lewis^x$, Sialyl $Lewis^a$ antigen is absent on most peripheral blood cells (Takada et al., 1991) and poorly expressed in serum and therefore, is unlikely to induce MHC restricted cellular immune response

## *4.2. Mechanism of action of therapeutic mAbs*

We proposed that injection of Ab1 directed against a selectin carbohydrate ligand into a host can inhibit the interaction between selectin and their ligands, and therefore, can inhibit the rolling of white blood cells on endothelial cells, and consequently leukocyte extravasation. Our proposal suggests that the injection of Ab1 will induce an anti-idiotypic antibody (Ab2) which will be an image of carbohydrate ligand and can bind to selectin molecule. Ab2 in its turn will induce anti-anti-idiotypic antibody (Ab3) which will be similar to Ab1 and to selectin molecule and can bind to the selectin carbohydrate ligand. As a result Ab1, Ab2 and/or Ab3 will be able to block selectin and/or their carbohydrate ligands.

Although $SLe^x$ is the main carbohydrate ligand involved in the interaction between selectins and immune cells, we have chosen to use as a therapeutic agent a mAb directed against $SLe^a$ antigen.

FACS analysis and fluorescence microscopy showed no binding of Ab1 to immune cells and since Ab3 is similar to Ab1 in terms of its binding activity, Ab3 is unlikely to bind to immune cells. Therefore, only Ab2 can act as competitive agent in blocking the carbohydrate ligand-selectin interaction through its binding to selectin molecule.

Animal experiments were conducted to evaluate the therapeutic efficacy of anti-SLe$^a$ mAb and to measure RAMA immune response. A correlation could be found between the RAMA immune response and the anti-inflammatory effect in the groups treated with HB1 mAb, and to a lesser extent in the groups treated with control mAb.

In vitro experiment studying the effect of Ab2 positive rat serum on leukocyte rolling showed a very good inhibition of leukocyte rolling with the HB1 mAb serum, and partial inhibition with the control mAb serum.

Results from animal experiment and in vitro experiment suggest that RAMA immune response is beneficial.

Although RAMA is not directed against molecules involved in immune cells extravasation, it may exert some therapeutic effect through binding to the Fc receptor on the surface of target cells and the induction of ADCC/CDC effect as illustrated in figure 43C.

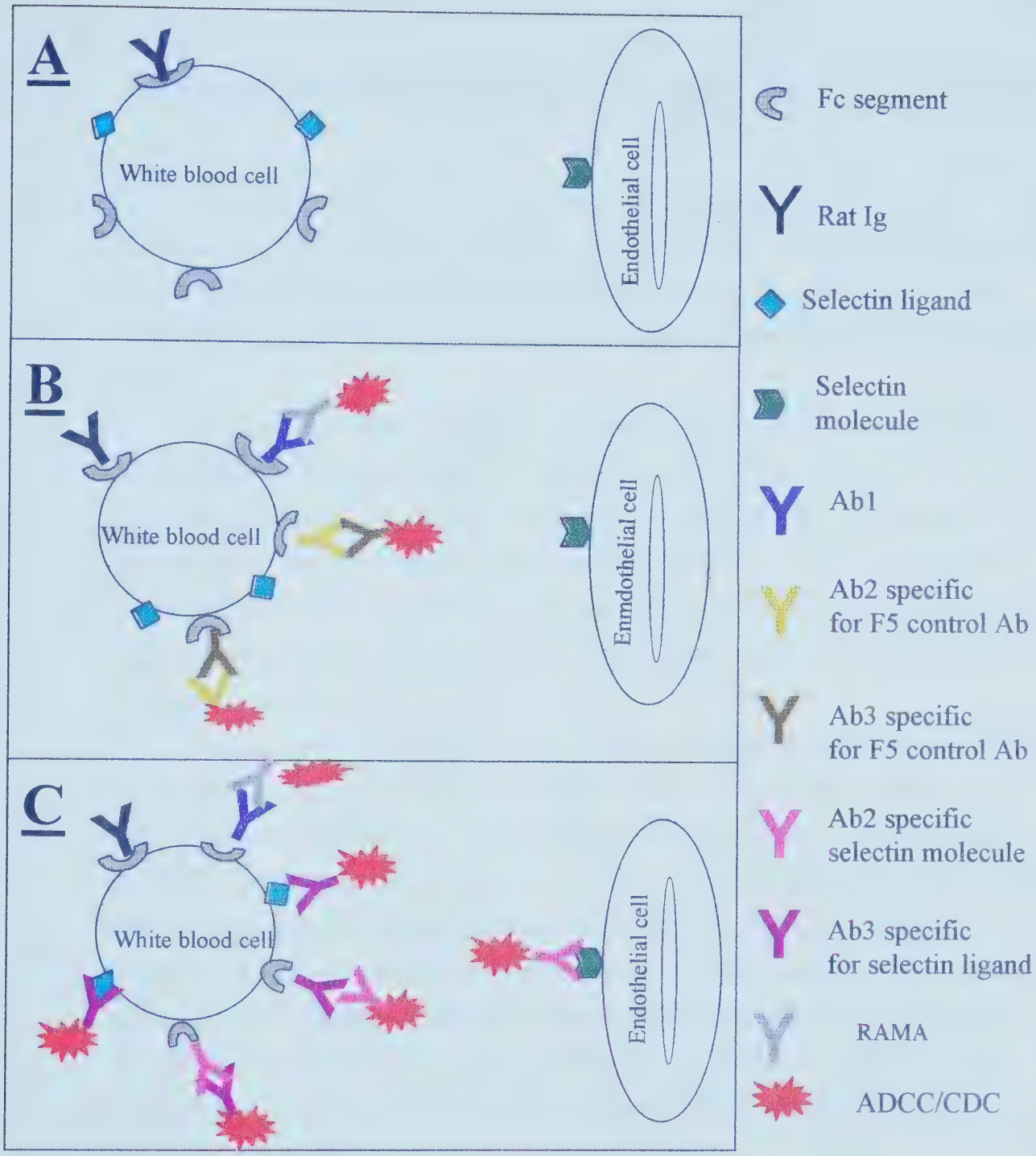



**Figure 43.** Illustration of the mechanism of action of responsible for the inhibition of leukocyte rolling on Endothelial cells. A, the mechanism of action in the group injected with PBS; B, the mechanism of action in the group treated with F5 control mAb; C, the mechanism of action in the group treated with the therapeutic HB1 mAb.

# CHAPTER 5. CONCLUSION AND FUTURE DIRECTIONS

The project has for objective the development of a new immunotherapeutic product for the treatment of autoimmune diseases. This project is based on the hypothesis that the inhibition of migration of immunocompetent cells to the site of inflammation would reduce or stop disease progression and considerably increase the quality of life for patients.

Adhesion molecules expressed on both immune cells and endothelial cells play crucial role in the migration of immunocompetent cells to the site of inflammation and the regulation of these molecules is known to play a significant role in modulating initiation, progression and severity of inflammation (McMurray, 1996). The importance of adhesion molecules in the control of leukocyte extravasation has made these molecules an attractive therapeutic target for the treatment of inflammatory disorders including autoimmune diseases.

Among adhesion molecules, selectins and their ligands are particularly attractive targets because they are involved in the rolling step of leukocytes that precedes and is essential for the extravasation of leukocytes into tissues in response to inflammatory stimuli (McIntyre et al., 1997). Number of studies have provided evidences showing that the inhibition of selectin function is an approach worth pursuing for the treatment of various inflammatory disorders (table 3). Because of their high specificity, monoclonal antibodies directed against selectin molecules, or their ligands represent a particularly attractive tool for the inhibition of selectin function. As a therapeutic agent, we have chosen to use a monoclonal antibody directed against $SLe^a$, a carbohydrate epitope recognized by selectins.

The main therapeutic application of the hypothesized anti-idiotypic anti-selectin approach is chronic inflammation. In fact, acute and chronic inflammation share the same mechanism of leukocyte migration for which selectin molecules are required. Therefore, we have in this study tested the $SLe^a$ specific monoclonal antibodies (HB1 and HB2) in an acute inflammation model using one of the most popular methods, carrageenan induced acute inflammation (edema) (Sánchez et al., 1998). Such an animal model is less time consuming and easier to handle than any chronic animal model.

Acute inflammation is very fast and strong response in nature, and is difficult to control or prevent. Despite these facts, in all experiments we obtained very promising results and it was consistently observed that the groups that have been treated with the anti-$SLe^a$ mAb, HB1 or HB2, have responded with the least inflammatory response measured by both caliper and water displacement measurements. In addition, in vitro experiment showed 100 % inhibition of PMNL rolling on selectin coated channels with RAMA positive HB1 rat serum.

Based on the results obtained from in vivo and in vitro experiments we proposed the following mechanisms: First, the anti-idiotypic Ab2 acts as a competitive agent in blocking carbohydrate ligand-selectin interaction through its binding to selectin molecule and is responsible for the inhibitory effect of HB1 serum on the PMNL rolling. Second, RAMA immune response may exert some therapeutic effect through the Fc receptor binding to target cells and the induction of ADCC/CDC effect as illustrated in figure 43C.The ADCC/ CDC effect which will cause toxic effect on cells, inhibition of cell function or cell rolling.

One could argue that $SLe^a$ is not the optimal target since $SLe^x$ is recognized to be the natural carbohydrate ligand for selectins. $SLe^a$ is an isomeric epitope of $SLe^x$ (Zhang et al., 1997) and is recognized by E and P-selectins (Paavonen and Renkonen, 1992) and may be by L-selectin (Paavonen and Renkonen, 1992).

The specificity of the therapeutic mAbs was chosen based on the following reasons: the approach can be patented and protected, the therapeutic mAb has already been characterized, and the development of an undesirable cellular immune response is unprobable. Although the evaluation of the therapeutic efficacy of a $SLe^x$ mAb is not the goal of this study it will be interesting to compare both $SLe^a$ and $SLe^x$ mAbs as potential drugs for chronic inflammation. In addition, studying the binding of $SLe^x$ and $SLe^a$ mAb to human serum by western blot analysis in order to identify the presence of circulating antigen needs to be performed. This kind of experiment will be particularly informative because as we described in section 3.2.6, the presence of circulating antigens may be important for the induction of cellular immune response which in this case will not be beneficial. An additional argument in favor of $SLe^a$ mAb over $SLe^x$ mAb utilization is that, in the case $SLe^a$ mAb Ab1 and Ab3 will not bind to neutrophils, while in the case of

$SLe^x$ mAb they will. The binding of Ab1 and Ab3 to neutrophils will result in more intense ADCC/CDC effect that may lead to neutropenia that is unfavorable effect for patients.

The results obtained with the utilization of HB1 mAb through an idiotypic network approach in acute inflammatory model are very encourging for further evaluation in chronic inflammatory models.

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